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TITLE: Elucidating the Role of cAb1 and the Abi-Family of cAb1  
Target Proteins in Cancer Development and Progression

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<b>13. ABSTRACT (Maximum 200 Words)</b> Abl-interactor (Abi) proteins bind and are phosphorylated by the non-receptor tyrosine kinases Abl and Arg. Two Abi family genes, Abi-1 and Abi-2, have been identified. Abi proteins demonstrate properties consistent with a potential tumor suppressor function. We investigated the role of Abi-1 and Abi-2 proteins and their interactions with c-Abl and c-Abl-derived oncogenes in normal development and tumorigenesis. Abi-1 and Abi-2 exhibit both unique and overlapping temporal-spatial patterns of expression during embryonic and post-natal mouse development. Abi-2 is enriched in regions of the central and peripheral nervous systems (CNS and PNS) pre- and post-natally. Abi-1 is also enriched in regions of the post-natal brain, but is not enriched in the pre-natal CNS and is absent from examined PNS structures. Abi proteins undergo changes in phosphorylation during development. Examination of Abi proteins in the presence of oncogenic forms of Abl and Src revealed loss of Abi expression due to ubiquitin-mediated degradation. Abi proteins were down-regulated or exhibited aberrant patterns of expression in some glioblastoma multiforme samples compared to normal brain tissue. Degradation has yet to be observed in other tumor settings, including breast cancer-derived cell lines overexpressing members of the erbB family of receptor tyrosine kinases. These findings support both unique and shared roles for Abi-1 and Abi-2 in mammalian development and suggest Abi degradation may be important in malignant transformation mediated by certain oncogenes.				
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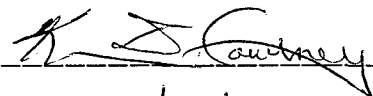
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## Introduction

Abl-interactor (Abi) proteins are substrates and adaptors for the non-receptor tyrosine kinases (NRTKs) Abl and Arg. Two Abi family genes, *Abi-1* (*e3B1*) and *Abi-2*, have been identified, both with multiple splice variants (1-5). We have cloned two isoforms of human Abi-2, designated Abi-2a and Abi-2b (Appendix A). Abi proteins interact with Abl and Arg through a carboxy-terminal SH3 domain and through proline-rich sequences. Abi proteins also contain a homeodomain homology region (HHR) with sequence similarity to the DNA binding domain of homeobox-containing proteins. Mutations in Abi-2 are capable of activating c-Abl transforming capacity, and overexpression of truncated Abi-1 in NIH3T3 cells suppresses v-Abl-mediated transformation (1, 2). Abi-1 has also been linked to cytoskeletal reorganization through its interactions with Sos-1 and Eps8, a substrate of several receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) (6). A complex of Abi-1, Sos-1, and Eps8 exhibits activity as a guanine nucleotide exchange factor for the Rac GTPase that regulates membrane ruffling and lamellipodia formation (6). Abi proteins are therefore linked to both receptor- and non-receptor tyrosine kinase- as well as GTPase- mediated signaling events. The purpose of this study is to investigate the role of Abi-family proteins and their interactions with the c-Abl tyrosine kinase and *c-Abl*-derived oncogenes in normal development and transformation. To provide a framework for the normal functions of Abi-1 and Abi-2, we have examined Abi expression, phosphorylation, and subcellular localization during embryogenesis and post-natal development. We have investigated the fate of Abi proteins in the presence of Abl- and Src- NRTK-derived oncogenes. Oncogenic forms of Abl and Src elicit the ubiquitin-mediated degradation of Abi proteins. We have compared Abi expression in paired human normal and tumorigenic tissue samples, as well as in breast cancer-derived cell lines that overexpress various erbB RTK family members. Ongoing studies are directed at identifying novel Abi-interacting proteins and examining the biological responses of cells to mutants of Abi proteins.

## Body

This summary describes the research accomplishments achieved as relate to the Tasks outlined in the Statement of Work for Grant Number DAMD17-98-1-8069.

### **Task 1: Determine the pattern of expression and tyrosine phosphorylation status of Abi-1, Abi-2, and c-Abl throughout mouse embryogenesis and post-natal development.**

Efforts to address this aim were summarized in the Annual Report submitted in July 1999. A portion of the work that was carried out to address this aim is presented in the appended article Courtney, K.D., Grove, M., Vandongen, H., Vandongen, A., LaMantia, S.-L., and Pendergast, A.M. (In Press) Localization and phosphorylation of Abl-interactor proteins, Abi-1 and Abi-2, in the developing nervous system. *Molecular and Cellular Neuroscience*.

### **Task 2: Identify novel binding partners for Abi-2 by use of a yeast two-hybrid screen.**

As stated in the Annual Report submitted in July 1999, an additional aim that arose from our initial studies was to identify proteins capable of interacting with Abi-1 and Abi-2. We sought to identify proteins that interact with the homeodomain homologous region (HHR) of Abi-2b, one of the isoforms of Abi-2 that arises from alternative splicing. The HHR is highly conserved among Abi proteins from *Drosophila* to humans (1,2,7). This domain shares 40-50% homology with the DNA-binding region of several homeobox-containing proteins (1,8). A number of homeodomain proteins exhibit protein-binding activity toward other proteins (9,10). For example, Hox-family homeodomain proteins have been shown to bind one another as well as other homeodomain proteins such as Pbx1 (9,10). We therefore hypothesized that the HHR of Abi proteins could mediate protein-protein interactions. Preliminary experiments performed with a glutathione S-transferase (GST) fusion of the hAbi-2b HHR (GST-HHR) suggested that this region is indeed capable of protein binding. GST-HHR bound to proteins from <sup>35</sup>S-labeled Bosc 23 cells that did not co-precipitate with GST alone or with the amino-terminal portion of Abi-2b upstream of the HHR.

We proposed to identify novel Abi-2 binding partners by performing a yeast two-hybrid screen. Given the results of Abi-2 expression studies (see Task 1 above), we chose to identify potential hAbi-2b HHR binding proteins expressed in the brain. Abi-2b HHR was subcloned into the pAS1-CYH2 vector for use in screening a human brain cDNA library from Clontech (Palo Alto, CA) for interacting proteins. The yeast two-hybrid screen makes use of the ability of yeast that harbor an interaction between a bait protein and a binding partner from the cDNA library to activate both nutritional and colorimetric reporter genes (11). The reporters are the *lacZ* gene, which confers a blue phenotype, and the growth selection reporter *HIS3*, which enables growth on media lacking histidine. These reporters are under the transcriptional control of the Gal4 upstream activation sequence. The Gal4 DNA-binding domain is tethered to the bait (HHR), while the transactivation domain is linked to species in the cDNA library.



Consequently, transcription activation of the reporters is dependent upon binding of the bait with an interacting protein in the cDNA library in order to bring the Gal4 DNA-binding and transactivation domains into proximity. This method was used to identify cDNA clones that are positive for interaction with the hAbi-2b HHR.

CG1945 yeast were co-transformed with the bait protein, a fusion of the Gal4 DNA binding domain and Abi-2b HHR, and the target, a fusion of the Gal4 activation domain and library cDNAs. The target library used in the co-transformation was amplified and contained  $7.5 \times 10^6$  clones, of which  $1.34 \times 10^5$  clones were co-transformed. 30 colonies were selected for having activated both the *lacZ* and *HIS3* reporters. To test the bait dependence of this transactivation, the positive colonies were grown in the presence of cycloheximide, which selects against the presence of the bait. Of these, 26 colonies showed bait dependence, requiring both the bait and the target to confer positive selection. The library plasmid expressing the potential hAbi-2b interacting protein was rescued from 18 of the 26 colonies and transformed by electroporation into *E. coli*. The target cDNAs from these positive clones have been isolated and are being sequenced. Positive interactions will be independently confirmed and the sites of interaction determined via *in vitro* and *in vivo* binding assays. Following identification of positive clones from the yeast two-hybrid screen by sequence analysis, GST-fusions will be prepared with the identified binding proteins (hereafter, "X") and used to test for binding of full-length  $^{35}\text{S}$ -labeled Abi proteins prepared by *in vitro* transcription and translation in reticulocyte lysates. Similar studies will be performed with lysates from cells that endogenously express Abi-2 species; Abi binding will be detected by immunoblot with antibodies against Abi proteins. *In vivo* binding to Abi-2b will be tested by overexpressing Abi-2b and "X" in 293T cells and immunoprecipitating with anti-Abi polyclonal antibody or antibody to epitope-tagged "X" and immunoblotting for both species. Biological assays relevant to the particular proteins identified will subsequently be performed.

**Task 3: Ascertain whether Abi protein expression or phosphorylation status is altered in cancer.**

Efforts to address this aim were summarized in the Annual Report submitted in July 1999. (A portion of the work that was carried out to address this aim is presented in the article Dai, Z., Quackenbush, R.C., Courtney, K.D., Grove, M., Cortez, D., Reuther, G.W., and Pendergast, A.M. (1998) "Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway" *Genes & Development* 12:1415-1424.)

**Task 4: Investigate the biological response of cells to mutants of Abi proteins.**

As indicated in the introduction, multiple splice variants have been identified for Abi-1 and Abi-2 (1-5). Two splice variants of human Abi-2 are Abi-2a and Abi-2b (Appendix A). To better understand the functions of Abi-1, Abi-2a, and Abi-2b, we are generating several mutants of each of these proteins for expression and localization within the cell. Several Abi-2a deletion mutations have previously been generated and cloned into mammalian expression vectors (1). These include Abi-2a( $\Delta$ 1-157), which eliminates the

amino-terminal 157 residues of Abi-2a that include the HHR and the first Abl-binding site; Abi-2a( $\Delta$ 1-100), which partially deletes the HHR; Abi-2a( $\Delta$ 244-401), a carboxy-terminal truncation that eliminates PEST sequences which are rich in Ser/Thr, Glu/Asp, and Pro residues, the polyproline region, and the SH3 domain; and Abi-2a( $\Delta$ SH3). Overexpression of Abi-2a( $\Delta$ 1-157) in NIH3T3 cells was shown to result in c-Abl activation and a transformed phenotype (1). Analogous mutations are being introduced into Abi-1 and Abi-2b, as well as mutations that specifically eliminate the HHR or the polyproline region. The latter has been identified as a binding site for Eps8 and related proteins (4,12,Courtney, K.D. and Pendergast, A.M., unpublished data). As noted in the introduction, Abi-1 has been linked to cytoskeletal reorganization through its interactions with Sos-1 and Eps8, a substrate of several receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) (6). A complex of Abi-1, Sos-1, and Eps8 exhibits activity as a guanine nucleotide exchange factor for the Rac GTPase that regulates membrane ruffling and lamellipodia formation (6). A number of the proposed mutants have been completed, in preparation for introduction into cells. EYFP-tagged mutants are being prepared in mammalian expression vectors for transient transfection and stable retroviral infection to enable analysis of effects on localization, morphology, viability, migration, and adhesion.

**Key Research Accomplishments:** as described for the Tasks provided in the Body of this application.

Task 1: Determine the pattern of expression and tyrosine phosphorylation status of Abi-1, Abi-2, and c-Abl throughout mouse embryogenesis and post-natal development.

- Localized *abi-1* and *abi-2* transcripts by *in situ* hybridization throughout mouse development
- Determined Abi and c-Abl protein levels and phosphorylation status at different stages of mouse development employing techniques of immunoprecipitation, Western blotting, and phosphatase treatment
- Subcellular localization of endogenous Abi, c-Abl, and Arg proteins in rat brain fractions by Western blot analysis
- Subcellular localization of enhanced yellow fluorescent protein (EYFP) tagged Abi-1 and Abi-2 in cultured fibroblasts and neurons by transfection, immunocytochemistry, and fluorescent and confocal microscopy techniques

Task 2: Identify novel binding partners for Abi-2 by use of a yeast two-hybrid screen.

- Identification of the homeodomain homologous region of hAbi-2b as a domain capable of mediating protein-protein interactions
- Isolation of 18 positive clones from a two-hybrid screen representing potential interacting proteins for hAbi-2b

Task 3: Ascertain whether Abi protein expression or phosphorylation status is altered in cancer. (Work done in collaboration with Zonghan Dai, Ph.D. and Robert Quackenbush, M.D.)

- Determined Abi proteins are degraded in a ubiquitin-dependent manner by Bcr-Abl and v-Src
- Confirmed by RNase protection and RT-PCR assays that Abi-1 and Abi-2 transcripts are generated in the presence of Bcr-Abl
- Observed Abi protein degradation in Ph<sup>1</sup>-positive leukemia cells, but not in Ph<sup>1</sup>-negative leukemias or in human breast, colon, lung, or liver tumors
- Observed down-regulation or aberrant expression patterns of Abi proteins in some glioma and glioblastoma multiforme samples relative to normal brain tissue
- Abi proteins were not degraded in untreated breast cancer derived cell lines overexpressing various erbB receptor tyrosine kinases

Task 4: Investigate the biological response of cells of various types, including myeloid, neuronal, normal breast epithelial and breast cancer-derived cells to mutants of Abi-2.

- Preparation of a panel of mutants of Abi-1 and Abi-2 for use in both transient transfection and infection to generate stable lines

## Reportable Outcomes:

### I. Manuscripts

1. Courtney, K.D., Grove, M., Vandongen, H., Vandongen, A., LaMantia, S.-L., and Pendergast, A.M. (2000) Localization and phosphorylation of Abl-interactor proteins, Abi-1 and Abi-2, in the developing nervous system. *Molecular and Cellular Neuroscience* In Press.
2. Quackenbush, R.Q., Reuther, G.W., Miller, J.P., Courtney, K.D., Pear, W.S., and Pendergast, A.M. (2000) Analysis of the biologic properties of p230 Bcr-Abl reveals unique and overlapping properties with the oncogenic p185 and p210 Bcr-Abl tyrosine kinases. *Blood* 95:2913-2921.
3. Dai, Z., Quackenbush, R.C., Courtney, K.D., Grove, M., Cortez, D., Reuther, G.W., and Pendergast, A.M. (1998) Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway. *Genes & Development* 12:1415-1424.

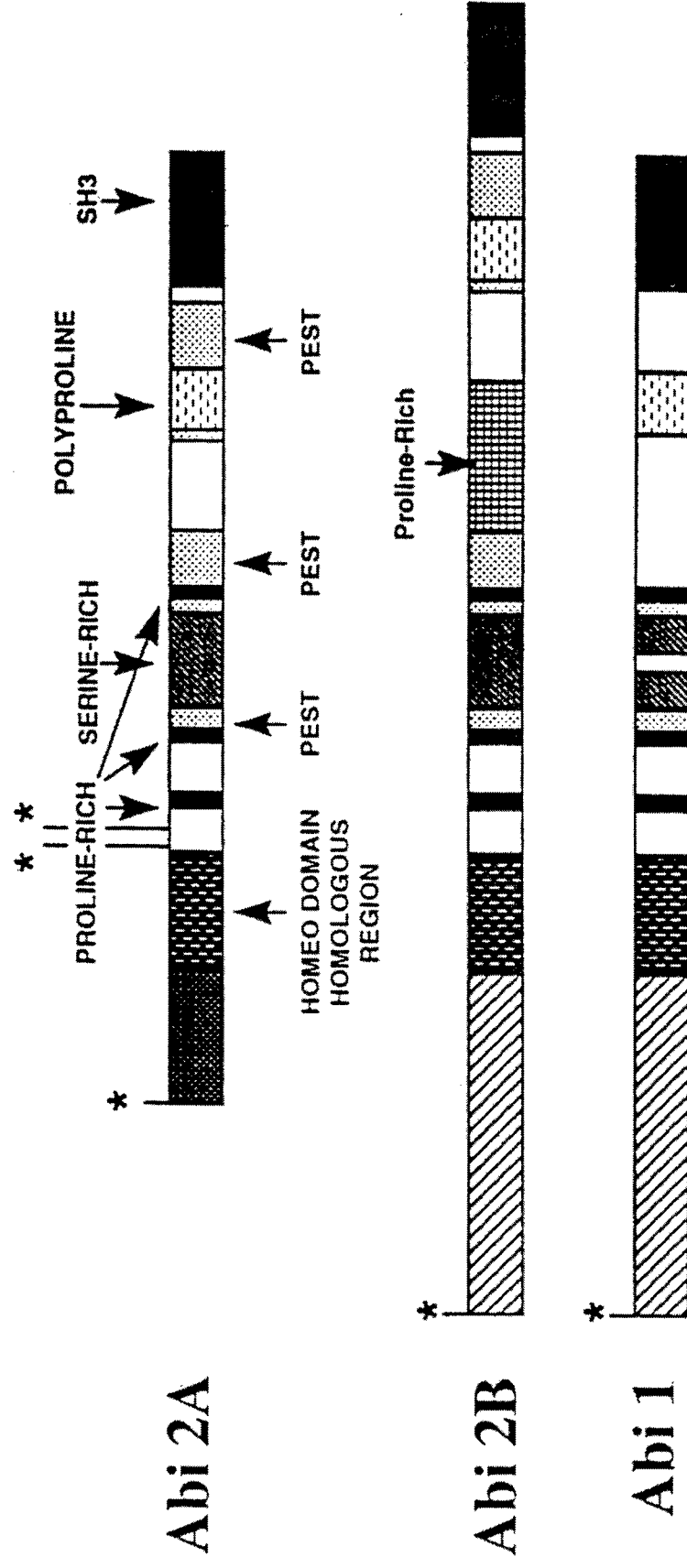
### II. Abstracts / Presentations:

1. Courtney, K.D., Dai, Z., Quackenbush, R.Q., Grove, M., Vandongen, H., Vandongen, A., LaMantia, A.-S., and Pendergast, A.M. Characterizing the Abl-interactor proteins, Abi-1 and Abi-2, and their roles in normal development and cancer progression. Presented at the Breast Cancer Research Program *Era of Hope* Meeting, June 8-11, 2000.
2. Courtney, K.D., Zipfel, P.A., Grove, M., Freneau, R.T., LaMantia, A.-S., and Pendergast, A.M. Expression of the Abl-interactor (Abi) genes and the Abl proto-oncogene during mouse embryogenesis and post-natal development. Presented at the Fourteenth Annual Meeting on Oncogenes, June 24-27, 1998.

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# Appendix A: Multiple Abi proteins may be expressed in human cells



Appendix A. Abi proteins contain multiple domains. Abi-2a and Abi-2b are splice variants of the same gene.

## CHARACTERIZING THE ABL-INTERACTOR PROTEINS, ABI-1 AND ABI-2, AND THEIR ROLES IN NORMAL DEVELOPMENT AND CANCER PROGRESSION

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Abl-interactor (Abi) proteins bind and are phosphorylated by the non-receptor tyrosine kinases Abl and Arg. Two Abi family genes, *Abi-1* and *Abi-2*, have been identified. Abi proteins demonstrate properties consistent with a potential tumor suppressor function. We investigated the role of Abi-1 and Abi-2 proteins and their interactions with c-Abl and c-Abl-derived oncogenes in normal development and tumorigenesis. Abi-1 and Abi-2 exhibit both unique and overlapping temporal-spatial patterns of expression during embryonic and post-natal mouse development. Abi-2 is enriched in regions of the central and peripheral nervous systems (CNS and PNS) pre- and post-natally. Abi-1 is also enriched in regions of the post-natal brain, but is not enriched in the pre-natal CNS and is absent from examined PNS structures. Abi proteins undergo changes in phosphorylation during development. Overexpression of fluorescent-tagged Abi-1 and Abi-2 proteins in fibroblasts and primary neurons reveals a cytosolic, punctate localization. Examination of Abi proteins in the presence of oncogenic forms of Abl and Src revealed loss of Abi expression due to ubiquitin-mediated degradation. Also, Abi proteins were down-regulated or exhibited aberrant patterns of expression in some glioblastoma multiforme samples compared to normal brain tissue. Degradation has yet to be observed in other tumor settings, including breast cancer-derived cell lines overexpressing members of the erbB family of receptor tyrosine kinases. These findings support both unique and shared roles for Abi-1 and Abi-2 in mammalian development and suggest Abi degradation may be important in malignant transformation mediated by certain oncogenes.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8069 supported this work.

## EXPRESSION OF THE ABL-INTERACTOR (ABI) GENES AND THE ABL PROTO-ONCOGENE DURING MOUSE EMBRYOGENESIS AND POST-NATAL DEVELOPMENT

Kevin D. Courtney, Patricia A. Zipfel, Matthew Grove, Robert T. Freneau, Anthony-Samuel LaMantia, and Ann Marie Pendergast

We have previously identified and cloned an Abl-interactor protein, Abi-2, which binds to and is phosphorylated by the c-Abl tyrosine kinase. Both Abi-2 and the related Abi-1 protein bind to c-Abl and to the Abl-related (Arg) tyrosine kinase. Abi-1 and Abi-2 are the products of different genes. Significantly, Abi-1 and Abi-2 have been shown to regulate the transforming capacity of Abl proteins. The roles of c-Abl and Abi proteins in normal cell growth and differentiation remain poorly understood. The objective of this study was to examine the expression of c-Abl and Abi proteins during mouse embryogenesis and post-natal development. Immunoprecipitation and Western blot analysis identified c-Abl and Abi proteins in mouse embryo lysates as early as embryonic day 10 (E10). Levels of c-Abl decreased as embryos approached full gestation. Abi proteins show increasing amounts of higher molecular weight forms with progression from E10-E13. Although Abi proteins were ubiquitously expressed, they were highly expressed in the brain. Abi protein expression was greater in lysates derived from embryonic heads than from trunks. *In situ* hybridization revealed *abi-1* transcripts to be prominent throughout the central nervous system (CNS) during embryonic and post-natal development. *Abi-2* message also appeared enriched in the CNS and peripheral nervous system throughout development, with apparent concentrations in specific neuronal populations, including dorsal root ganglia at E16, the Purkinje cell layer of the cerebellum, and Ammon's horn and the dentate gyrus of the hippocampus. A downward shift in apparent molecular weight with increasing age was observed for Abi proteins detected in brain lysates prepared from late embryonic and post-natal mice. This shift to faster migrating forms with age corresponded in part with loss of phosphorylation of Abi proteins in brain lysates from older mice. In cultured mouse embryonal carcinoma P19 cells, Abi protein mobility was altered following treatment with retinoic acid, which induces neuronal differentiation. These data suggest that Abi expression may be developmentally regulated and that Abi proteins may play a role in c-Abl mediated signal transduction in the brain.



## Localization and Phosphorylation of Abl-Interactor Proteins, Abi-1 and Abi-2, in the Developing Nervous System

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Abl-interactor (Abi) proteins are targets of Abl-family non-receptor tyrosine kinases and are required for Rac-dependent cytoskeletal reorganization in response to growth factor stimulation. We asked if the expression, phosphorylation, and cellular localization of Abi-1 and Abi-2 supports a role for these proteins in Abl signaling in the developing and adult mouse nervous system. In mid-to late-gestation embryos, *abi-2* message is elevated in the central and peripheral nervous systems (CNS and PNS). *Abi-1* mRNA is present, but not enhanced, in the CNS, and is not observed in PNS structures. Abi proteins from brain lysates undergo changes in apparent molecular weight and phosphorylation with increasing age. In the postnatal brain, *abi-1* and *abi-2* are expressed most prominently in cortical layers populated by projection neurons. In cultured neurons, Abi-1 and Abi-2 are concentrated in puncta throughout the cell body and processes. Both Abi and Abl proteins are present in synaptosomes and growth cone particles. Therefore, the Abi adaptors exhibit proper expression patterns and subcellular localization to participate in Abl kinase signaling in the nervous system.

### INTRODUCTION

The Abl-interactor proteins, Abi-1 (e3B1) and Abi-2, are common downstream targets of the Abl-family of nonreceptor tyrosine kinases (NRTKs), which includes c-Abl and Arg (Dai and Pendergast, 1995; Shi *et al.*,

1995; Wang *et al.*, 1996a; Biesova *et al.*, 1997). Abi proteins bind to and are substrates of c-Abl and Arg tyrosine kinases and are implicated in the regulation of cell growth and transformation (Dai and Pendergast, 1995; Shi *et al.*, 1995; Wang *et al.*, 1996a; Dai *et al.*, 1998). Abi-1 has also been linked to cytoskeletal reorganization through its interactions with Sos-1 and Eps8, a substrate of several receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) (Scita *et al.*, 1999). A complex of Abi-1, Sos-1, and Eps8 exhibits activity as a guanine nucleotide exchange factor for the Rac GTPase that regulates membrane ruffling and lamellipodia formation (Scita *et al.*, 1999). Microinjection of fibroblasts with anti-Abi-1 antibodies resulted in abrogation of Rac-dependent membrane ruffling in response to platelet derived growth factor (PDGF) stimulation (Scita *et al.*, 1999). Abi proteins are therefore linked to both receptor- and nonreceptor tyrosine kinase- as well as GTPase-mediated signalling events.

Abi-1 and Abi-2 share significant identity, exhibiting greater than 90% conservation in their amino-termini and in their carboxy-terminal SH3 domains. Multiple isoforms of both proteins have been identified, resulting from alternative splicing events (Biesova *et al.*, 1997; Taki *et al.*, 1998). Interactions with c-Abl and Arg tyrosine kinases are mediated through the SH3 domain and proline-rich regions of Abi-1 and Abi-2 (Dai and Pendergast, 1995; Shi *et al.*, 1995; Wang *et al.*, 1996a). Abi-1 and Abi-2 also contain a homeobox-homology region (HHR) that retains several critical residues of the helix-turn-helix DNA-binding motif common to home-

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odomains (Dai and Pendergast, 1995; Shi *et al.*, 1995). *Abi* genes are widely expressed in mice and humans, with highest mRNA levels observed in the brain (Dai and Pendergast, 1995; Shi *et al.*, 1995). *Abi-2* is the mammalian ortholog of the *Xenopus laevis xlan4* gene. *xlan4* is developmentally regulated, with increased transcript levels at the neurula stage localizing to dorsal axial structures, which are principally comprised of the developing CNS (Reddy *et al.*, 1992). In larvae and adults, *xlan4* is primarily expressed in the brain (Reddy *et al.*, 1992). *abi-2*, as well as the related *abi-1*, may therefore play a role in neuronal development and function.

Increasing evidence points to roles for Abl- and Src-family NRTKs in neuronal development and axonogenesis. Src-family kinases (including c-Src, Fyn, Lyn, and c-Yes) have been implicated in neuronal development, differentiation, and neurite outgrowth (Grant *et al.*, 1992; Beggs *et al.*, 1994; Ignelzi *et al.*, 1994). Src has been shown to regulate N-methyl-D-aspartate (NMDA) receptor activity, and Lyn and Fyn have been linked to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signaling and regulation (Yu *et al.*, 1997; Hayashi *et al.*, 1999; Narisawa-Saito *et al.*, 1999). Expression of either c-Abl or Arg is essential for proper neurulation in mice (Koleske *et al.*, 1998). Genetic studies of *Drosophila abl* (*D-abl*) have revealed roles for *D-abl* in axonogenesis and growth cone pathfinding (Hu and Reichardt, 1999; Van Etten, 1999).

Little is known regarding potential common targets of the c-Abl, Arg, and Src tyrosine kinases in neuronal development. *Abi* proteins are functionally linked to Abl and Src-family tyrosine kinases. *Abi-1* and *Abi-2* have been shown to regulate the transforming capacity of Abl proteins, and expression of oncogenic forms of Abl and Src tyrosine kinases downregulates *Abi* protein levels (Dai and Pendergast, 1995; Shi *et al.*, 1995; Dai *et al.*, 1998). *Abi* proteins may function as targets for c-Abl and Arg tyrosine kinases in neuronal cells. To this end, we examined and compared the expression patterns of *abi-1* and *abi-2* in the developing nervous system, and the subcellular localization of *Abi*- and Abl-family proteins in neurons. Our results show that *abi-1* and *abi-2* exhibit unique expression patterns in early CNS and PNS development, but similar localization in the postnatal brain. Moreover, *Abi* adaptor proteins are expressed with c-Abl and Arg in the neuron cell body, at synapses, and in growth cone particles, where they may function to transduce signaling events downstream of the Abl kinases or may contribute to the regulation of c-Abl and Arg kinase activity.

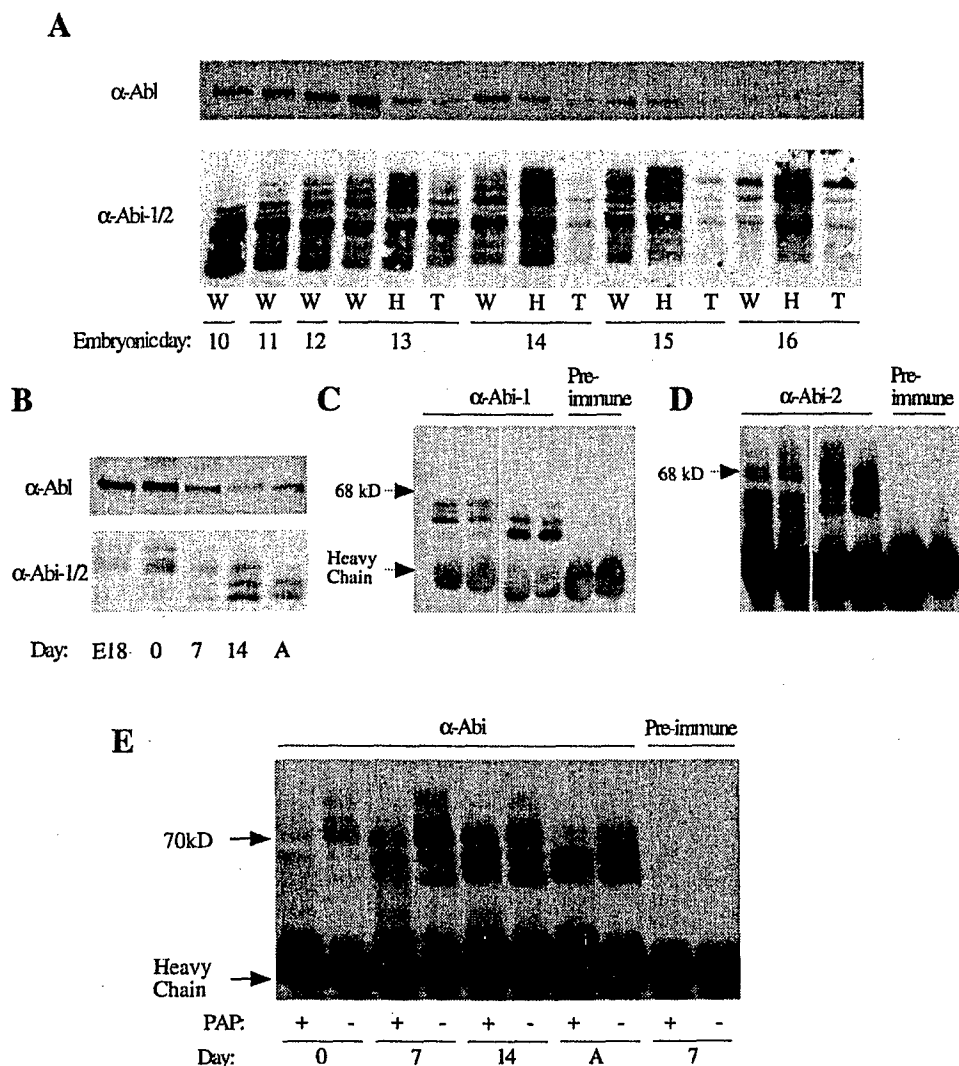
## RESULTS

### *Abi* Protein Expression during Embryogenesis and Postnatal Brain Development

We first asked whether *Abi-1*, *Abi-2*, and c-Abl proteins are present in developing and adult tissues, including the CNS. Lysates of mouse embryos of gestational age 10–16 days (E10–E16) were prepared and analyzed by immunoprecipitation and Western blotting techniques. Peak c-Abl expression is observed at the earliest ages examined (E10–E13), consistent with previous reports of *c-abl* transcript levels (Müller *et al.*, 1982) (Fig. 1A). Using anti-*Abi* serum 5421, which recognizes the protein products of both *abi-1* and *abi-2* (Dai *et al.*, 1998), multiple *Abi* protein bands are recognized from E10–E16 (Fig. 1A). c-Abl and *Abi* proteins are more highly expressed in lysates prepared from embryo heads than trunks. Among postnatal tissues examined, *Abi* protein is most highly expressed in the brain (data not shown). In late embryonic and postnatal brain lysates, *Abi* proteins undergo a shift in apparent molecular weight to faster migrating forms on reducing gels with increasing age of mice (Fig. 1B). c-Abl expression diminishes in postnatal brain lysates in older mice (Fig. 1B).

The multiple bands and changing apparent molecular weight observed for *Abi* proteins in Western blots of mouse embryo and postnatal brain lysates may reflect differences in expression of *Abi-1* and *Abi-2*, multiple alternative splice variants of both *Abi-1* and *Abi-2*, or changes in posttranslational modifications of these proteins. To determine whether there are differences in the expression of *Abi-1* and *Abi-2* at different ages, we generated antibodies which specifically recognize *Abi-1* or *Abi-2* by immunoprecipitation (M. Grove, R. C. Quackenbush, and A. M. Pendergast, unpublished observations). Both *Abi-1* and *Abi-2* are expressed in embryos and in post-natal brains, with *Abi-1*- and *Abi-2*-specific antibodies recognizing multiple bands corresponding to *Abi-1* or *Abi-2*, respectively (Figs. 1C and 1D). Both *Abi* proteins undergo a marked shift in mobility over time in postnatal brain lysates. The multiple bands observed in Western blots and immunoprecipitation experiments are specific for the *Abi* proteins, as confirmed by loss of *Abi-2* protein bands in lysates prepared from brains of *abi-2*<sup>-/-</sup> mice, with retention of *Abi-1* protein expression (M. Grove and A. M. Pendergast, unpublished data).

Changes in phosphorylation of *Abi* proteins could contribute to the observed shifts in protein mobility. In this regard, *Abi-1* has been shown to become hyper-



**FIG. 1.** Abi and c-Abl proteins are expressed during embryogenesis, and Abi proteins undergo changes in phosphorylation and apparent molecular weight in the developing postnatal brain. (A, B) c-Abl and Abi proteins are detected in E10-E16 mouse embryos and in E18 and postnatal brain lysates. Lysates (30  $\mu$ g of tissue per lane) were electrophoresed on SDS-PAGE gels, transferred to nitrocellulose membranes, and blotted with anti-Abi serum 5421 or a monoclonal antibody recognizing c-Abl. (A) c-Abl expression decreases in embryos late in gestation (top). Abi proteins are expressed from E10-E16, with higher protein levels observed in head (H) fractions than in embryo trunks (T) (bottom). W, whole embryo lysate. (B) c-Abl levels appear higher in late embryonic (E18) and neonatal (0, 7 days) brain lysates than in brains from older mice (14 days; A, adult) (top). Abi proteins undergo an apparent molecular weight shift in the developing postnatal brain (bottom). (C, D) Lysates (2 mg total protein) of embryos (E12, E14) or postnatal brains (7 days; A, adult) were immunoprecipitated with antibodies recognizing Abi proteins or with normal rabbit serum (Pre-immune). (C) Immunoprecipitation of embryo and brain lysates with anti-Abi-1-specific rabbit polyclonal antibodies or with preimmune serum reveals a shift in the mobility of Abi-1. (D) Immunoprecipitation of embryo and brain lysates with anti-Abi-2-specific polyclonal antibody reveals that Abi-2 is shifted to lower molecular weight forms between P7 and adult mice. (E) PAP treatment of Abi proteins immunoprecipitated from postnatal brain lysates leads to changes in protein migration on reducing gels, consistent with dephosphorylation of Abi proteins in neonatal brain lysates.

phosphorylated on serine following mitogenic stimulation of serum-starved fibroblasts that overexpress EGFR (Biesova *et al.*, 1997). To test whether phosphorylation of Abi proteins contributes to the observed changes in apparent molecular weight, immunoprecipi-

tated Abi-1 and Abi-2 proteins from brain lysates were treated with potato acid phosphatase (PAP) or calf intestinal alkaline phosphatase (CIP). PAP (Fig. 1E) or CIP (data not shown) treatment elicits a marked shift in the electrophoretic migration of the highest molecular

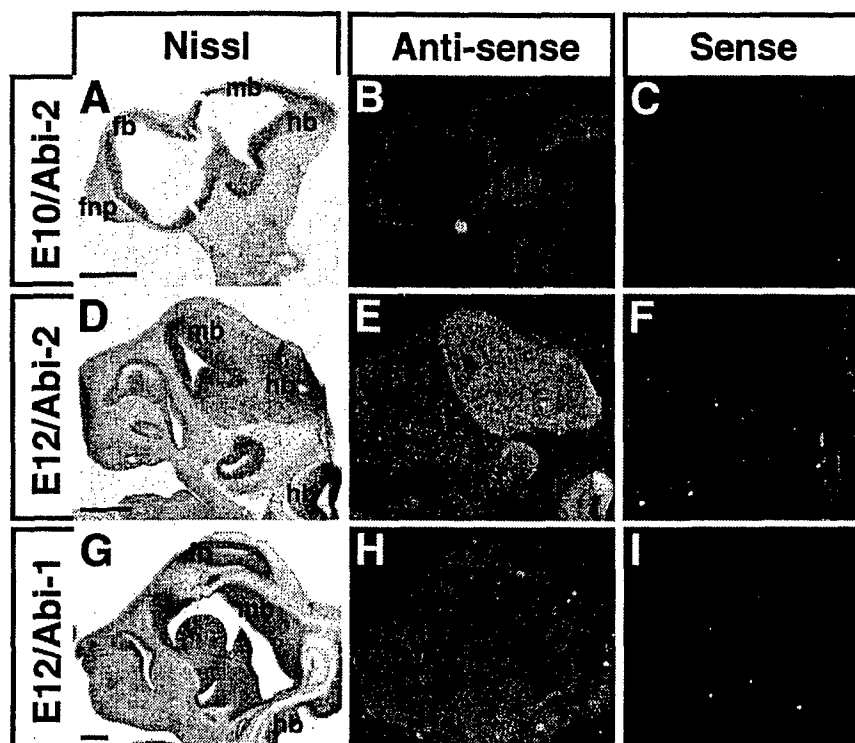


FIG. 2. *abi-2* expression in mouse embryos at E10 and E12 is concentrated in the CNS. *In situ* hybridization was carried out with sense and antisense probes to *abi-1* and *abi-2* on sagittal sections of E10 and E12 mouse embryo heads. (A–C) *abi-2* message at E10 is concentrated in the frontonasal prominence (fnp) and the neuroepithelium of the forebrain (fb), midbrain (mb), and hindbrain (hb) of the E10 mouse embryo. *abi-2* hybridization by (B) antisense and (C) sense probes is presented. (D–F) *abi-2* message is prominent in the CNS at E12. (E) Antisense and (F) sense riboprobes were used. (G–I) *abi-1* expression is uniform throughout sagittal sections of E12 mouse embryo heads. Hybridization by (H) antisense and (I) sense probes for *abi-1* is presented. (A, D, G) Bright field images are shown. Scale bars, 1 mm.

weight forms of Abi proteins in P0 and P7 brain lysates, as well as changes in the mobility of Abi proteins in lysates from older mice. PAP treatment of postnatal brain lysates reveals changes in Abi-2 phosphorylation in lysates from neonates compared to brains of older mice, while changes in Abi-1 phosphorylation are not observed by this method (data not shown). Because c-Abl levels diminish with age in post-natal brain lysates, we hypothesized that c-Abl-mediated tyrosine phosphorylation might contribute to the observed differences in Abi phosphorylation at different ages. However, we do not detect tyrosine phosphorylation of Abi proteins by Western blot of anti-Abi immunoprecipitates from postnatal brain lysates with anti-phosphotyrosine antibody 4G10 (data not shown).

#### Expression of *abi-1* and *abi-2* during Early Differentiation of the Nervous System

To more precisely localize Abi-1 and Abi-2 expression during development, we prepared frozen sections

of mouse embryos and postnatal brains. Abi-1 and Abi-2 proteins could not be detected by immunohistochemistry using available Abi-1 and Abi-2 antibodies. We therefore performed *in situ* hybridization with sense and antisense probes specific for *abi-1* and *abi-2* transcripts.

We first examined *abi-1* and *abi-2* expression prior to the onset of cortical neurogenesis. At E10 the neuroepithelium of the presumptive forebrain is undergoing symmetrical cell division resulting in a population of pluripotent progenitor cells (Zindy *et al.*, 1997). At E10, *abi-2* mRNA detected with an antisense riboprobe appears more highly expressed in the neuroepithelium of the developing forebrain, midbrain, and hindbrain regions of the CNS, as well as in the frontonasal prominence, than in the adjacent cephalic and trunk mesenchyme (Fig. 2B). This elevated expression can be best appreciated by comparing the antisense probe-labeled section with the section labeled with an *abi-2* sense riboprobe (Figs. 2B and 2C). Although there is a slight amount of detectable labeling in the sense probe-la-

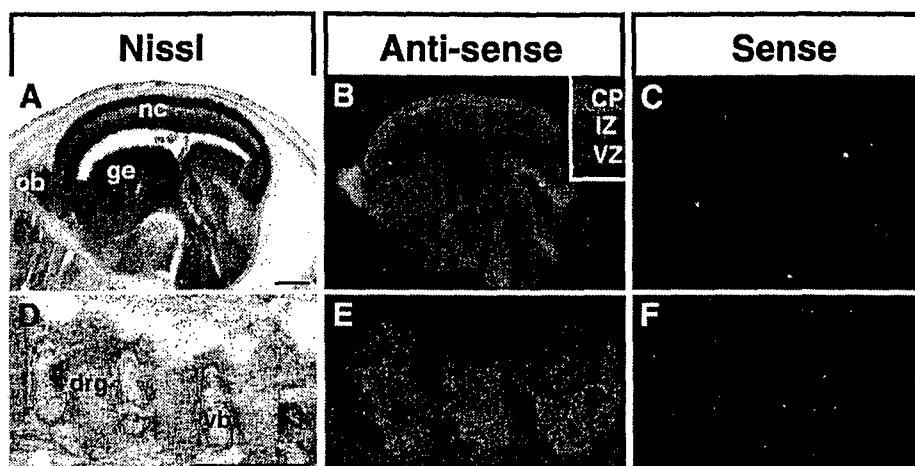


FIG. 3. *abi-2* message is enhanced in the CNS and PNS at E16. (A–C) Sagittal sections of E16 mouse embryo heads reveal prominent *abi-2* expression in the developing CNS. (B) *abi-2* levels as determined by hybridization with an antisense riboprobe appear higher in the cortical plate (CP) than in the underlying intermediate zone (IZ) or ventricular zone (VZ) (B, inset) and are elevated in the olfactory bulb (ob). ge, ganglionic eminence; nc, neocortex. (C) *abi-2* hybridization by a sense riboprobe is shown. (D–F) *abi-2* is prominently expressed in dorsal root ganglia (drg). vb, vertebral body. (E) Antisense and (F) sense riboprobes were used. (A, D) Bright field images are presented. Scale bars, 0.5 mm.

beled section, it is uniform throughout the embryo. Accordingly, *abi-2* expression is apparently enhanced in the developing brain at mid-gestation. *Abi-1* message is present in the developing CNS at E10, but does not appear more prominent in the neuroepithelium compared to the surrounding tissue (data not shown). Significantly, c-Abl and Arg are also prominently expressed in the neuroepithelium at E9–E10.25 (Koleske *et al.*, 1998).

By E12, following the onset of neurogenesis, *abi-2* message is enhanced throughout the developing CNS (Figs. 2D–2F). Elevated *abi-2* is detected in the developing brain and along the full length of the spinal cord (data not shown). *abi-1* does not appear to be enhanced in the CNS relative to other tissues at E12 (Figs. 2G–2I). At E16 *abi-2* hybridization remains enhanced throughout the CNS, but with apparent regional distinctions (Figs. 3A–3C). *Abi-2* message is particularly prominent in the olfactory bulb at this stage (Fig. 3B). Within the developing neocortex, *abi-2* mRNA is more highly expressed in the cortical plate than in the underlying intermediate zone (IZ) or ventricular zone (VZ) (Fig. 3B, inset). Enhanced expression of *abi-2* is not limited to the CNS. In the periphery, *abi-2* levels are also elevated in dorsal root ganglia (DRGs) at E16 (Figs. 3D–3F). At E16 hybridization to *abi-1* mRNA is detected throughout the CNS in an unrestricted fashion which is not elevated relative to surrounding tissues, similar to the expression pattern observed at E12 (data not shown). In contrast to *abi-2*, which is expressed in DRGs (Fig. 3E), *abi-1*

is not detected in these PNS structures (data not shown).

#### *abi-1* and *abi-2* Expression in the Postnatal Brain

To determine whether *abi-1* and *abi-2* show prominent expression in specific regions of the postnatal brain, we performed *in situ* hybridization on brain sections from postnatal day 7 (P7) mice. At this stage of development, only specific neuronal populations continue to proliferate, including the cells of the dentate gyrus and the external granular layer of the cerebellum (Meller and Glees, 1969). While differences in expression between *abi-1* and *abi-2* are observed in the embryonic brain, *abi-1* and *abi-2* exhibit similar hybridization patterns in the P7 brain, with strongest expression in the cerebral cortex, hippocampus, dentate gyrus, olfactory bulb, and cerebellum (Figs. 4A–4F). The elevated expression of both genes in the cerebral cortex does not appear to be confined to particular layers. Similar expression patterns were observed for *abi-1* and *abi-2* in brain sections from P0 mice (data not shown).

The expression patterns of *abi-1* and *abi-2* in the brains of adult mice (>6 weeks) are similar to the patterns observed in the P7 brain. *Abi-2* transcripts are prominently expressed in the neocortex, hippocampus, and dentate gyrus (Figs. 5A and 5B). Again, *abi-2* message does not appear to be limited to particular layers of the neocortex (data not shown). In the cerebellum, *abi-2* appears highest in the Purkinje layer (Figs. 5E–5G). The

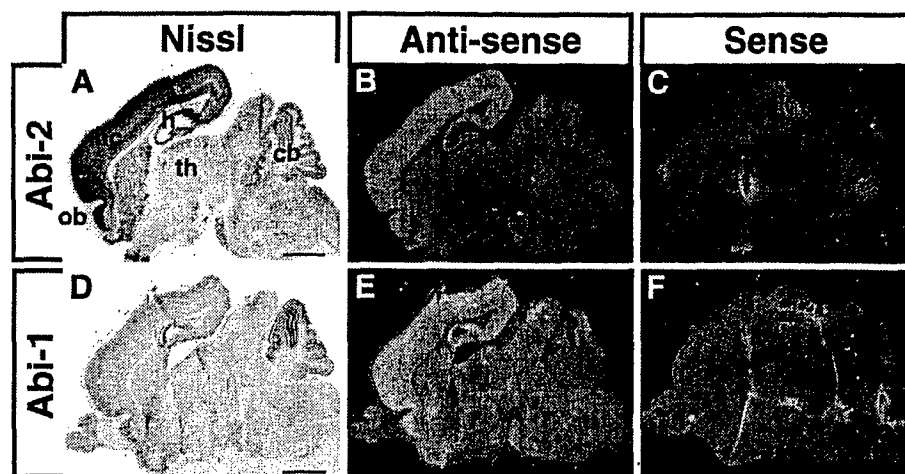


FIG. 4. *abi-1* and *abi-2* mRNAs are enhanced in specific regions of the mouse brain at postnatal day 7. Sagittal sections of brains from P7 mice were analyzed by *in situ* hybridization with probes against *abi-1* and *abi-2* mRNAs. (A–C) *abi-2* is elevated in the neocortex (nc), hippocampus (h), and cerebellum (cb). th, thalamus. (D–F) *abi-1* hybridization is also prominent in the neocortex, hippocampus, olfactory bulb, and cerebellum. Sections were hybridized with (B, E) antisense and (C, F) sense riboprobes. (A, D) Bright field images are shown. Scale bars, 2 mm.

mitral cell layer of the olfactory bulb also shows prominent *abi-2* hybridization (Figs. 5K–5M). Similar to *abi-2*, *abi-1* transcripts are expressed in the hippocampus and dentate gyrus (Figs. 5C and 5D). *abi-1* mRNA is also detected in the neocortex (data not shown). In the cerebellum, both the Purkinje layer and the granular layer show *abi-1* hybridization above background (Figs. 5H–5J). Like *abi-2*, *abi-1* is also prominent in the mitral cell layer of the olfactory bulb (Figs. 5N–5P).

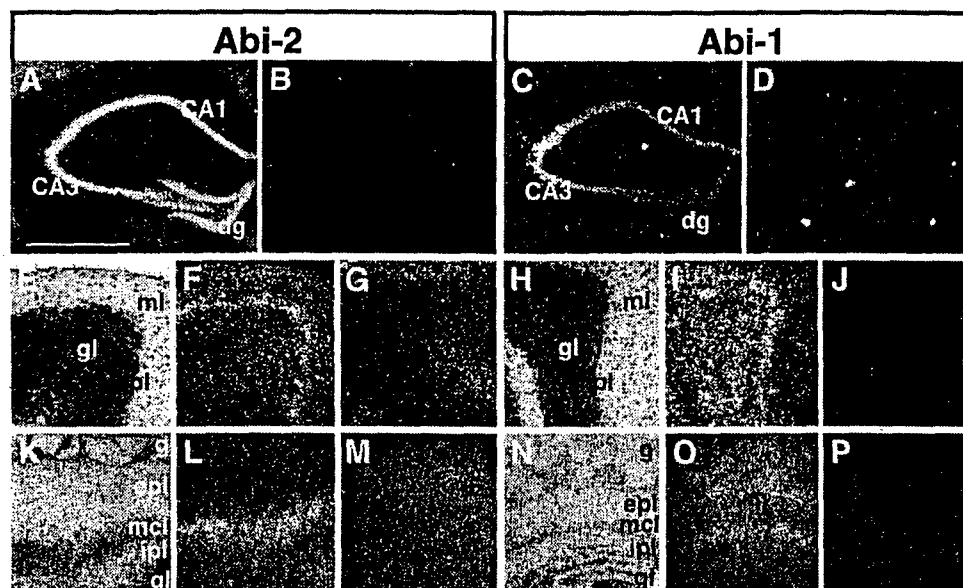
#### Subcellular Localization of Fluorescently Tagged *Abi-1* and *Abi-2* in Cultured Neurons

Having identified the regional distribution of *abi-1* and *abi-2* messages in the nervous system at different developmental stages, we wished to examine the subcellular localization of Abi-1 and Abi-2 proteins within neurons. To localize Abi-1 and Abi-2 proteins within living cells, we generated fusion proteins of isoforms of Abi-1 and Abi-2 with enhanced yellow fluorescent protein (EYFP) and expressed these constructs in neurons and glial cells cultured from embryonic day 18 (E18) rat hippocampal tissue.

We first examined Abi-1 · EYFP and Abi-2 · EYFP expression in neurons transfected within a few days of plating (3–6 days in culture) and imaged live neurons the day after transfection. Neurons in culture for 2–6 days have extended axons and are undergoing rapid dendritic outgrowth and the completion of neurite differentiation (Dotti et al., 1988; Pennypacker et al., 1991).

Abi-1 · EYFP and Abi-2 · EYFP exhibit a punctate pattern of expression in neurons transfected at 5–6 days in culture (Figs. 6B and 6C). This pattern of fluorescence is most prominent in the cell body (Figs. 6B and 6C, insets), but also extends into neurites and is suggestive of vesicular structures. Neurons transfected at 5 days in culture with EYFP alone do not exhibit the punctate distribution of fluorescence associated with Abi expression (Fig. 6A).

To localize Abi-1 · EYFP and Abi-2 · EYFP in mature neurons, we transfected neurons after 14 days in culture. Neurons reach maturation, with differentiated axons and dendrites, by 7 days in culture (Dotti et al., 1988). Two weeks after plating, the neurons in this *in vitro* culture system have established multiple synaptic contacts and have extended long, thin axons that can be distinguished from dendrites (Dotti et al., 1988). Abi-1 · EYFP expression is again most prominent in the cell body, where it retains the vesicular pattern observed in younger neurons (Fig. 6E, inset). This punctate pattern extends into multiple dendrites, where Abi-1 · EYFP is observed within the length of the dendrite, as well as at discrete points that appear to be associated with dendritic spines (Figs. 6E and 6F). Abi-1 · EYFP fluorescence is enhanced in association with a subset of spines, suggesting Abi-1 may concentrate at specific synapses (Fig. 6F, arrows). Abi-1 · EYFP is also present in the axon (Fig. 6E). The punctate localization of Abi-1 · EYFP is distinct from that of EYFP alone, which is expressed diffusely in the cell body and neurites (Fig. 6D). Under



**FIG. 5.** *abi-1* and *abi-2* are concentrated in specific regions of the adult mouse brain. (A, B) Hybridization with (A) antisense and (B) sense probes to *abi-2* reveals expression in the CA1 and CA3 regions of the hippocampus and the dentate gyrus (dg) in >6-week-old mice. (C, D) Hybridization with (C) antisense and (D) sense probes to *abi-1* shows expression in the hippocampus and dentate gyrus. (E–G) (E) Bright-field and (F, G) dark-field images of a cerebellar folium are shown. (F) *abi-2* appears elevated in the Purkinje layer (pl) of the cerebellum by antisense hybridization. gl, granular layer; ml, molecular layer. (G) Hybridization with a sense control probe is shown. (H–J) *abi-1* appears concentrated in the Purkinje layer and the granular layer. (H) A bright field image is shown. (I, J) Dark-field images are provided for *abi-1* hybridization with (I) antisense and (J) sense probes. (K–M) *abi-2* appears concentrated in the mitral cell layer (mcl) of the olfactory bulb. epl, external plexiform layer; g, glomerulus; gl, granular layer; ipl, internal plexiform layer. (K) Bright-field and (L, M) dark-field images are shown. *abi-2* hybridization by (L) antisense and (M) sense probes is presented. (N–P) *abi-1* also appears elevated in the mitral cell layer of the olfactory bulb. (N) Bright-field and (O, P) dark-field images are provided. Scale bars, 0.5 mm.

similar culture and transfection conditions, we were unable to detect expression of Abi-2 · EYFP in neurons transfected after 2 weeks in culture.

Arg, the predominant Abl-family kinase in the brain, has previously been localized to the cytosol in neuroepithelial cells and transfected fibroblasts (Wang and Kruh, 1996; Koleske *et al.*, 1998). To examine whether Abi-1 · EYFP and Abi-2 · EYFP colocalize with the Arg kinase, transfected neurons were fixed and imaged by confocal microscopy following immunocytochemistry. Confocal microscopy revealed that endogenous Arg, Abi-1 · EYFP, and Abi-2 · EYFP are excluded from the nucleus in transfected neurons (data not shown). Endogenous Arg expression is observed throughout the cytosol in the cell body and extends into neurites. The punctate pattern of Abi-1 · EYFP and Abi-2 · EYFP expression colocalizes in part with Arg in the cell body and in neurites; however, overexpression of the Abi proteins does not sequester endogenous Arg protein into identically localized, discrete punctate structures (data not shown).

#### **Subcellular Localization of Endogenous Abi-1, Abi-2, and Abl-Family Kinases in Fractionated Brain Lysates**

We next localized endogenous Abi proteins within neuronal cell compartments. Because we were unable to localize Abi proteins within cultured neurons or tissue sections by indirect immunofluorescence using our anti-Abi antibodies, we examined endogenous Abi protein expression in lysates of fractions prepared from neonatal and adult rat brains by Western blotting with anti-Abi serum 5421. Fractionation of adult rat brain yielded the postnuclear supernatant and synaptosomes (synaptic terminals) (Strack *et al.*, 1997). Arg has previously been shown to be enriched in synaptosomes (Koleske *et al.*, 1998). Like Arg, the c-Abl tyrosine kinase and Abi adaptor proteins are expressed in synaptosomes (data not shown). To determine whether mammalian Abi and Abl-family proteins localize to growth cones, brains from neonatal (P3) rats were fractionated to yield growth cone particles and growth cone membranes (Patterson and Skene, 1999). A greater fraction of



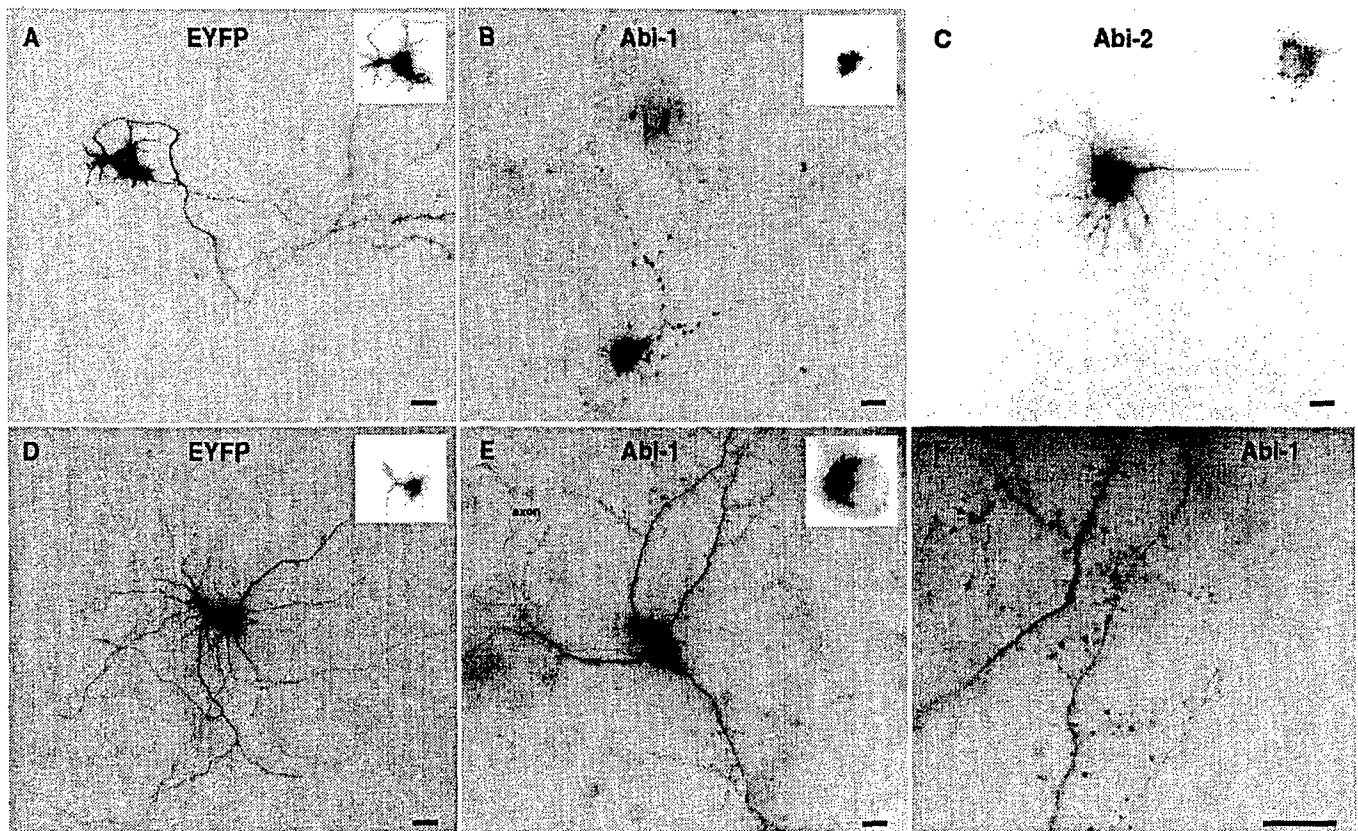


FIG. 6. Abi-1·EYFP and Abi-2·EYFP localize with a punctate distribution in the cell body and neurites of cultured neurons. (A–C) E18 rat hippocampal neurons cultured for 5–6 days were transfected with (A) EYFP, (B) Abi-1·EYFP, or (C) Abi-2·EYFP and imaged live by fluorescence microscopy. (A) EYFP is expressed throughout the neuron and appears uniformly distributed in the cell body (inset). (B) Abi-1·EYFP exhibits a punctate distribution in the cell body (inset) and in neurites. (C) Abi-2·EYFP expression also yields a punctate distribution in the cell body (inset) and in neurites. (D–F) Neurons cultured for 2 weeks were transfected with (D) EYFP or (E, F) Abi-1·EYFP. (D, inset) EYFP is again expressed throughout the neuron. (E) Abi-1·EYFP can be seen in dendrites and the axon (labeled). Abi-1·EYFP is expressed in a punctate pattern in the cytosol (inset). Abi-1·EYFP also localizes to structures that appear to be dendritic spines. (F) Magnification of (E) showing Abi-1·EYFP expression in dendrites and apparent dendritic spines (arrows). Scale bars, 20  $\mu$ m.

the Abi proteins is present in growth cone particles and growth cone membrane fractions compared to the supernatant (Fig. 7). Abl and Arg kinases are also present in these fractions, consistent with previous results which have localized *Drosophila* Abl to the growth cone (Henkemeyer et al., 1987; Gertler et al., 1989). Similarly, Src is enriched in growth cone preparations compared to the supernatant as previously reported (Bixby and Jhabvala, 1993). Thus, Abi proteins may be involved in NRTK signaling in the growth cones.

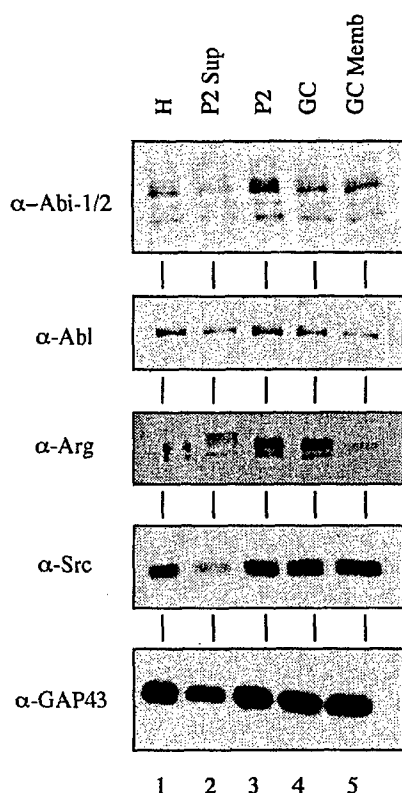
## DISCUSSION

Mounting evidence implicates Abl- and Src-family NRTKs in nervous system development and neuronal

function. Abi-family proteins have previously been identified as substrates and binding partners of c-Abl and Arg and as targets for degradation mediated by oncogenic forms of Abl and Src (Dai and Pendergast, 1995; Shi et al., 1995; Dai et al., 1998). Here we report the temporal and spatial distribution of *abi-1* and *abi-2* in the developing nervous system and the subcellular localization of Abi proteins in neurons. Our findings suggest that Abi-1 and Abi-2 may perform unique and shared functions in the nervous system that change during development. Changes in phosphorylation and apparent molecular weight of Abi proteins during CNS maturation suggest Abi-1 and Abi-2 may participate in developmentally regulated signaling events in neuronal cells.

Abi and c-Abl proteins are expressed in the CNS





**FIG. 7.** Abi, c-Abl, and Arg proteins are expressed in growth cone particles. Lysates of postnatal day 3 rat forebrain subcellular fractions (10  $\mu$ g total protein) were analyzed by Western blotting with antibodies against the Abl family kinases c-Abl and Arg, Abi proteins, Src, and growth cone associated protein (GAP43). Sequential fractionation of the whole forebrain homogenate (H) yields the supernatant (P2 sup) and pellet fractions (P2) following low-speed centrifugation and growth cone particles (GC) and growth cone membrane fractions (GC Memb) following high-speed centrifugation through Ficoll gradient (Patterson and Skene, 1999). Abi, c-Abl, and Arg proteins appear enriched in the P2 and GC fractions over the P2 supernatant.

throughout development. Multiple bands corresponding to Abi-1 and Abi-2 proteins are observed by Western blot analysis of embryo and postnatal brain lysates. These results are consistent with previous work that has identified multiple splice variants for Abi-1 and Abi-2 (Wang *et al.*, 1996a; Biesova *et al.*, 1997; Taki *et al.*, 1998; Ziemnicka-Kotula *et al.*, 1998). Additionally, Abi proteins are modified posttranslationally by phosphorylation (Fig. 1E and Biesova *et al.*, 1997) and ubiquitination (Dai *et al.*, 1998). These modifications increase the complexity of the protein migration pattern associated with Abi expression.

The localization of *abi-1* and *abi-2* transcripts was identified throughout nervous system development. Our results show *abi-2* expression is most prominent in

the CNS in embryos at E10 and E12 and throughout the CNS and in DRGs at E16. Like *abi-2*, Abl and Arg kinases are most highly expressed in the neuroepithelium at E9.5–10.25 (Koleske *et al.*, 1998). Although *abi-2* message is present throughout the developing neocortex at E16, it is more highly expressed in the region of postmigratory, differentiated neurons and glia that defines the cortical plate than in the migrating population of cells in the underlying intermediate zone or in the dividing neuroblasts of the ventricular zone (Angevine and Sidman, 1961). In contrast, *abi-1* does not show CNS enrichment in embryos and is not observed in DRGs. By postnatal day 0, *abi-1* and *abi-2* are highly expressed in similar regions of the brain, and this pattern is maintained in older mice. Sites of prominent *abi-1* and *abi-2* expression include the hippocampus, the dentate gyrus, the neocortex, the mitral cell layer of the olfactory bulb, and the Purkinje layer of the cerebellum. Abi-2 therefore may play a role in PNS development that is not shared by Abi-1, and Abi-1 and Abi-2 may have unique functions in early CNS development. The regional concentration of *abi-1* in the postnatal brain, not apparent in the embryo, suggests a late gestational or neonatal onset of possible neural cell-specific functions for Abi-1.

The localization of Abi-1 and Abi-2 within postmitotic neurons suggests that they may contribute to multiple processes in these cells. Expression of Abi-1 · EYFP and Abi-2 · EYFP in cultured neurons less than 1 week after plating yields a vesicular staining pattern that extends into neurites and is excluded from the nucleus. In neurons cultured for 2 weeks, Abi-1 · EYFP is predominantly somatodendritic and appears concentrated in a subset of dendritic spines. Abi-1 · EYFP is also observed in the axon. Although transfection of fluorescent-tagged Abi-1 and Abi-2 into hippocampal cultures permitted observation of both Abi-1 and Abi-2 neuronal distribution *in vivo* in immature neurons, we were unable to successfully transfect more mature neurons with Abi-2 · EYFP. Perhaps Abi-2 protein is more unstable than Abi-1, or Abi-2 overexpression is toxic to these cells.

Abi-1 · EYFP and Abi-2 · EYFP distribute to multiple neuronal compartments. We wished to confirm this localization by examining endogenous Abi proteins using subcellular fractionation techniques. Following fractionation of neonatal and adult brains, endogenous c-Abl, Arg, and Abi proteins are observed in growth cone particles and at synaptic terminals. Abi proteins may therefore impinge upon Abl-family kinase functions in these structures. Studies in *Drosophila* have revealed effects of D-Abl during axonogenesis and growth cone pathfinding. *D-abl*<sup>-/-</sup> flies exhibit premature arrest of

intersegmental nerve b (ISNb) growth cones leading to defective axon outgrowth (Wills *et al.*, 1999). Furthermore, additional *Drosophila* mutants have been characterized that link *D-abl* to axon outgrowth, including mutants for the D-Abl substrate enabled (Ena) and the actin binding protein Chickadee (Profilin) (Gertler *et al.*, 1995; Wills *et al.*, 1999).

Rac and other Rho-family GTPases are regulators of growth cone movement and directional guidance (Luo *et al.*, 1994, 1996; Threadgill *et al.*, 1997; Kaufmann *et al.*, 1998). Recent work has shown a role for Abi-1 in cytoskeletal reorganization mediated by Rac (Scita *et al.*, 1999). Microinjection of fibroblasts with antibodies to Abi-1 blocks membrane ruffling in response to PDGF (Scita *et al.*, 1999). Interestingly, the same phenotype is also observed in *abl*<sup>-/-</sup> cells (Plattner *et al.*, 1999). In the adult rat brain, *rac1* message is elevated in areas of synaptic plasticity, including the hippocampus, dentate gyrus, and granule and Purkinje cells of the cerebellum (Olenik *et al.*, 1997). *rac1* expression in the adult rat brain largely coincides with expression of *abi-1* and *abi-2* in the adult mouse brain. Thus, Abi proteins may colocalize with Rac1 in the brain and may participate in regulation of Rac function in neurons as has been proposed in fibroblasts (Scita *et al.*, 1999). Abi adaptors may integrate signaling pathways regulated by NRTKs and small GTPases at sites of dynamic cytoskeletal remodeling.

Our data suggest that Abi proteins are both pre- and postsynaptic. Sites of *abi-1* and *abi-2* enrichment in the postnatal brain correspond to projection neuron populations (e.g., mitral cells, cerebellar Purkinje cells) and regions which exhibit synaptic plasticity (Maness, 1992; Zhang *et al.*, 1997). Interestingly, recent work has shown an interaction between Abi-1 and the cytoskeletal protein erythroid spectrin (Ziemnicka-Kotula *et al.*, 1998). Erythroid-type brain spectrin has been shown to exhibit regulated binding to NMDA receptors, which are key components of long term potentiation and synaptic plasticity (Wechsler and Teichberg, 1998). Future work will examine whether Abi proteins functionally interact with NMDA receptors.

Abi proteins undergo changes in phosphorylation and are shifted in reducing gels during post-natal brain development, suggesting Abi involvement in signaling events that may promote neuron differentiation and development. It is possible that the observed changes in Abi phosphorylation coincide with a decrease in the population of proliferating cells in the brain or with the attenuation of specific mitogenic signals. Abi-1 has previously been shown to become hyperphosphorylated on serine following mitogenic stimulation by serum or

EGF treatment of serum-starved fibroblasts overexpressing EGFR (Biesova *et al.*, 1997). Significantly, we have recently shown that the c-Abl kinase is activated by the binding of the growth factors EGF and PDGF to receptor tyrosine kinases, and that this activation is mediated in part by Src-family kinases (Plattner *et al.*, 1999). Although we were unable to detect Abi tyrosine phosphorylation in brain lysates (data not shown), Abl-family kinases may participate in the generation of signals leading to Abi phosphorylation.

Although a number of proteins that interact with and are substrates for mammalian c-Abl or D-Abl have been proposed to contribute to Abl function in neural development, neuronal targets of Arg have not been previously identified (Van Etten, 1999). While c-Abl and Arg share significant sequence identity in the amino-termini, the large carboxy-terminal regions of these proteins are largely divergent. However, the site of Abi SH3 binding is conserved in the C-terminus of c-Abl and Arg (Perego *et al.*, 1991). Thus, Abi-1 and Abi-2 are excellent candidates for participating in c-Abl- and Arg-mediated events in neurons and neuronal precursors. Abl-family kinases may transduce signals from multiple receptors present in neuronal cell bodies, at synapses, or in growth cones. Abi proteins may serve as regulators or downstream targets of Abl-family kinases at each of these sites. Homozygous deletion of *abi-1* and *abi-2* in mice will enable us to further examine these possibilities. Studies are underway to ascertain the effects of loss of *abi-1* and *abi-2* on mouse neuronal development and function.

## EXPERIMENTAL METHODS

### Antibodies

Rabbit polyclonal anti-Abi-1 sera 6987 and 6988 were raised against the internal Abi-1 peptide HGNNQ-PARTGTLSTNP. Rabbit polyclonal anti-Abi-2 antibody 7887 was raised against the internal Abi-2 peptide RFKVSTQNMKMGGLPRTTPPT. Rabbit polyclonal antibody anti-Abi 5421 has been described previously (Dai *et al.*, 1998). Monoclonal antibody 21-63 against c-Abl has been previously described (Schiff-Maker *et al.*, 1986). Mouse anti-Abl monoclonal antibody 8E9 was purchased from Pharmingen (San Diego, CA). Mouse monoclonal anti-phosphotyrosine antibody clone 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). A polyclonal antibody recognizing Arg was generously provided by Dr. A. Koleske (Department of Molecular Biophysics and Biochemistry, Yale

University, New Haven, CT). A polyclonal antibody recognizing c-Src and HRP-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-GAP43 antibody was purchased from Boehringer-Mannheim (Indianapolis, IN). A monoclonal antibody recognizing synaptophysin was purchased from Sigma (St. Louis, MO). Rhodamine-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Pierce (Rockford, IL). HRP-conjugated Protein A was purchased from Amersham Pharmacia (Arlington Heights, IL).

### Tissue Preparations

Tissue lysates for protein analysis were prepared as follows. Embryos from timed-pregnant CD-1 mice were dissected free of extraembryonic membranes and homogenized in ice cold RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) in the presence of protease and phosphatase inhibitors (10  $\mu$ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM  $\text{Na}_3\text{VO}_4$ ) (Gertler *et al.*, 1996). Brain extracts were similarly prepared. Lysates were clarified by centrifugation, and protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA).

For *in situ* hybridization, embryos were prepared in one of two ways. Following dissection free of extraembryonic membranes, embryos either were immersed immediately in 2-methylbutane at  $-40^\circ\text{C}$  or were fixed overnight at  $4^\circ\text{C}$  in 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline (PBS). Fixed embryos were subjected to a sucrose gradient (10–30% sucrose in PBS) and embedded in 3.5% agar/3.5% sucrose and OCT. Sections of 16–25  $\mu\text{m}$  thickness were cut on a Reichert Jung cryostat, mounted on CSS-100 silylated slides (Cel Associates, Houston, TX), and stored at  $-80^\circ\text{C}$  until hybridization. Brains from post-natal mice were immersed in 2-methylbutane as described above or were dissected following perfusion of the mouse through the left ventricle of the heart with PBS followed by 4% PFA/4% sucrose. Following perfusion, brains were fixed and cryoprotected as described for embryos. Reagents used in preparation of tissues for *in situ* hybridization were pretreated with diethylpyrocarbonate (Sigma).

### Synthesis of RNA Probes for *abi-1* and *abi-2*

*Abi-2* sense and antisense probes were prepared by *in vitro* transcription from a linearized construct of pBlue-Script II Sk +/– plasmid (Stratagene, La Jolla, CA) into

which a 435 nucleotide (nt) fragment of *abi-2* mouse genomic DNA was subcloned. This fragment included 48 nt of intronic sequence and 397 nt from a single exon encompassing the SH3 domain and a portion of the 3' untranslated region (UTR) of murine *abi-2* (Dai *et al.*, 1998). *Abi-1* sense and antisense probes were prepared by *in vitro* transcription from either of two linearized templates. The first comprised a 373-nt cDNA fragment of murine *abi-1* spanning the SH3 domain and a portion of the 3' UTR subcloned into pGEM-T (Promega, Madison, WI). The second was composed of the pGEM-T plasmid containing a 635 nt cDNA fragment of the 5' end of murine *abi-1*.

### In Situ Hybridization

*In situ* hybridization with [ $^{35}\text{S}$ ]UTP-labeled probe (Dupont NEN, Boston, MA) was performed as described (Wang *et al.*, 1996b) with the following modifications. Following hybridization for 12–21 h at  $50^\circ\text{C}$  with 4000 cpm/ $\mu\text{l}$  [ $^{35}\text{S}$ ]UTP-labeled probe, sections were washed briefly in room temperature  $2\times$  SSC and then for 1 h in 4 L of  $2\times$  SSC at  $50^\circ\text{C}$  with stirring. Subsequent RNase treatment and washes were carried out as described, with the exception that the final wash was continued overnight at room temperature following 3 h at  $50^\circ\text{C}$ . Slides were exposed to Kodak NT/B2 emulsion (Eastman Kodak, Rochester, NY) at  $4^\circ\text{C}$  for 2 weeks–1 month. Developed slides were counterstained with Hematoxylin-Eosin Y or methyl green. Most lower magnification images were acquired with a Leica M420 Photomakroskop M400 microscope with dark- and bright-field illumination and attached Diagnostic Instruments Spot Videocamera. Images were processed in Adobe Photoshop 5.0. Photography of some lower magnification images was performed with a Wild Photomakroskop M400 microscope with dark- and bright-field illumination using Kodak TMAX 100 film. Photographic negatives were scanned into Adobe Photoshop 4.0 with a Polaroid SprintScan 35 Plus scanner. Higher magnification images were captured by a Leitz Ortholux microscope with a Dage-MTI CCD 72S camera.

### Analysis of Abl and Abi Proteins in Tissue Lysates

Western blot analysis to detect c-Abl or Abi proteins was carried out on 30  $\mu\text{g}$  of tissue lysate resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Anti-Abi serum 5421 and mouse monoclonal anti-Abl antibodies 21-63 or 8E9

were used. To examine specific expression of Abi-1 and Abi-2 proteins, lysates were incubated with anti-Abi-1-specific sera 6987 and 6988 or anti-Abi-2-specific serum 7887, respectively, for 6 h–overnight at 4°C. Lysates were subsequently incubated with protein A sepharose (PAS) beads (Amersham Pharmacia) for 1–1.5 h. Immunoprecipitates were washed twice with RIPA buffer in the presence of inhibitors (described above) and boiled in 2× SDS sample buffer. Proteins were resolved by SDS-PAGE and Western blot analysis was performed with anti-Abi-1 sera 6987 and 6988 or anti-Abi serum 5421.

To determine the phosphorylation status of Abi proteins in the mouse brain, lysates (2 mg total protein) were incubated for 2 h–overnight at 4°C with anti-Abi serum 5421, anti-Abi-1 sera 6987 and 6988, anti-Abi-2 serum 7887, or normal rabbit serum. Following incubation for 30 min–1 h with PAS beads, immunoprecipitates were washed extensively with RIPA buffer and inhibitors, followed by potato acid phosphatase (PAP) buffer (40 mM Pipes, pH 6.0, 1 mM DTT, 1 mM MgCl<sub>2</sub>, 10 µg/ml aprotinin). Immunoprecipitates bound to PAS beads were resuspended in PAP buffer in the presence or absence of 2 µg potato acid phosphatase (Boehringer Mannheim) as indicated, and incubated 10 min at 30°C. Following an additional wash with RIPA buffer and inhibitors, PAS beads were boiled with 2× SDS sample buffer and electrophoresed on SDS-PAGE gels. Subsequent Western analysis was performed as described above.

### Cell Culture and Transfection

Embryonic day-18 (E18) rat hippocampal tissue (BrainBits, Springfield, IL) was dissociated by gentle trituration and cells were plated on poly-D-lysine (50 mg/ml)-coated coverslips at a density of  $23 \times 10^3$  cells/cm<sup>2</sup>. Cells were grown in Neurobasal medium (Gibco-BRL), complemented with B27, 0.5 mM glutamine, and 25 mM L-glutamate, and maintained at 37°C in 5% CO<sub>2</sub>. The same medium (minus L-glutamate) was used for partial medium exchanges every 4 days. Hippocampal cells were transfected on the days indicated, using FuGENE 6 Reagent (Boehringer-Mannheim) as described in the package insert. In short, 2 µg of plasmid DNA was mixed with 4 µl of FuGENE 6 Reagent diluted in 100 µl of Neurobasal medium. This mixture was incubated at room temperature for 15 min and then added to the culture medium. Fluorescence images were obtained using a Micromax CCD camera (Princeton Electronics) attached to a Nikon Diaphot epi-fluorescence

microscope equipped with a FITC filter set (Chroma Technology Corp., Brattleboro, VT).

### Enhanced Yellow Fluorescent Protein (EYFP) Fusions

The Abi-1 coding region, minus the first methionine, and 123 nt of 3' UTR were amplified by PCR and subcloned into the plasmid pEYFP-C1 (Clontech, Palo Alto, CA) at the *Bgl*III site. The isoform of Abi-1 used to construct the Abi-1 · EYFP fusion was obtained from EST zr24a06.r1 (GenBank Accession No. AA232072, ATCC, Rockville, MD) (R. C. Quackenbush, and A. M. Pendergast, unpublished results). The Abi-2 coding region was amplified by PCR and subcloned into pEYFP-C1 at the *Bam*HI site. The isoform of Abi-2 used to construct the Abi-2 · EYFP fusion was obtained from EST yo44f11.s1 (GenBank Accession No. R87714, ATCC) (Z. Dai and A. M. Pendergast, unpublished results). EYFP was located at the N terminus of both constructs.

### Subcellular Fractionation of Neonatal and Adult Rat Brain Lysates

Neonatal (P3) rat brain fractions to yield growth cone particles were generously provided by Dr. J. H. P. Skene (Department of Neurobiology, Duke University Medical Center, Durham, NC) and were prepared as described (Patterson and Skene, 1999). Adult rat brain postnuclear supernatant and synaptosome fractions were the generous gift of Dr. R. Colbran (Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN) and were prepared as published (Strack et al., 1997).

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# Analysis of the biologic properties of p230 Bcr-Abl reveals unique and overlapping properties with the oncogenic p185 and p210 Bcr-Abl tyrosine kinases

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The reciprocal translocation between chromosomes 9 and 22 that fuses coding sequences of the *Bcr* and *Abl* genes is responsible for a remarkably diverse group of hematologic malignancies. A newly described 230-kd form of Bcr-Abl has been associated with an indolent myeloproliferative syndrome referred to as chronic neutrophilic leukemia. We have cloned the corresponding gene and examined the biologic and biochemical properties of p230 Bcr-Abl after retroviral-mediated gene transfer into hematopoietic cell lines and primary bone marrow cells. p230 Bcr-Abl-expressing 32D myeloid cells were fully growth factor-independent and activated similar signal

transduction pathways as the well-characterized p210 and p185 forms of Bcr-Abl. In contrast, primary mouse bone marrow cells expressing p230 required exogenous hematopoietic growth factors for optimal growth, whereas p185- and p210-expressing cells were independent of growth factors. The 3 Bcr-Abl proteins exerted different effects on differentiation of bone marrow cells. p185 induced outgrowth of lymphoid precursors capable of tumor formation in immunodeficient mice. In contrast, p210- and p230-expressing bone marrow cells caused limited outgrowth of lymphoid precursors that failed to form tumors in immunodeficient mice. Removal of cytokines and

autologous stroma from Bcr-Abl-expressing bone marrow cultures produced the expansion of distinct lineages by the various Bcr-Abl proteins. p185 drove expansion of cytokine-independent lymphoid progenitors, while p210 and p230 generated cytokine-independent monocyte/myeloid cells. These findings suggest that the different Bcr-Abl fusion proteins drive the expansion of different hematopoietic populations, which may explain the association of the various Bcr-Abl oncoproteins with different spectra of human leukemias. (Blood. 2000;95:2913-2921)

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## Introduction

The *Bcr-Abl* oncogene is formed by the reciprocal translocation between chromosomes 9 and 22 and is associated with diverse human leukemias.<sup>1</sup> The *Bcr-Abl* oncogene product, Bcr-Abl, is a chimeric protein with deregulated tyrosine kinase activity. Bcr-Abl exerts its leukemogenic effects via both autophosphorylation and phosphorylation of cellular substrates. This ultimately leads to activation of signal transduction pathways involved in the altered biologic behavior of Bcr-Abl-containing cells. These include the Ras, Raf, Erk, Jnk, Myc, Jak/Stat, PI3kinase-Akt, and NF- $\kappa$ B pathways, among others.<sup>2-12</sup> More recently, the oncogenic Bcr-Abl proteins have been shown to elicit the ubiquitin-dependent degradation of target proteins.<sup>13</sup>

The leukemia-associated *Bcr-Abl* oncogenes vary in the amount of the *Bcr* gene that is included within the chimera.<sup>1</sup> The structure of the various Bcr-Abl oncoproteins may influence the disease phenotype associated with their expression. p185 Bcr-Abl (also referred to as p190 Bcr-Abl) contains the dimerization, binding, SH2 and serine/threonine kinase domains of Bcr and is associated with the subset of acute lymphoblastic leukemias (ALL) that are

Philadelphia chromosome positive (Ph1<sup>+</sup>).<sup>14,15</sup> ALL is typically an acute, aggressive leukemia that requires prompt treatment with high-dose chemotherapy.<sup>16</sup> p210 Bcr-Abl contains additional *Bcr* sequences coding for Pleckstrin homology (PH) and Dbl-like domains and is associated with nearly all cases of chronic myelogenous leukemia (CML).<sup>17</sup> CML begins as a chronic myeloproliferative disorder that is amenable to management with chemotherapeutic or biologic agents, but inevitably progresses to a fatal blast crisis of either myeloid or lymphoid phenotype.

Recently, Pane et al<sup>18</sup> have shown that a subset of patients with Ph1<sup>+</sup> chronic neutrophilic leukemia (CNL) have a unique breakpoint (designated  $\mu$ ) within the *Bcr* gene on chromosome 22.<sup>19</sup> This breakpoint is 3' to the more common breakpoints found in patients with CML and the subset of patients with ALL that are Ph1<sup>+</sup>. Thus, p230 Bcr-Abl contains additional *Bcr* coding sequences that are not found in the p185 or p210 variants. Specifically, p230 Bcr-Abl contains the calcium-phospholipid binding (CalB) domain and the first third of the domain associated with GTPase activating activity for p21rac (GAP<sup>rac</sup>). CNL, which is also known in the literature as

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neutrophilic chronic myelogenous leukemia, is a rare disorder marked by sustained mature neutrophilic expansion with mild hepatosplenomegaly. This disease typically has a more indolent course when compared with classical CML, and progression to blast crisis is uncommon.<sup>18</sup> However, a few patients have been reported who exhibit typical CML and carry the  $\mu$  breakpoint that generates p230 Bcr-Abl.<sup>20-22</sup>

Thus, the unique protein domains that are contributed by the various Bcr breakpoints, when translocated to the *c-Abl* gene can lead to diseases with remarkably different phenotypes. To gain insight into the molecular pathology of the various Bcr-Abl-related leukemias, we have cloned the CNL-associated p230 Bcr-Abl oncogene and expressed the protein product in hematopoietic cell lines as well as primary mouse bone marrow cells under conditions that favor the outgrowth of either myeloid or lymphoid progenitors. Here, we analyze the biochemical and biologic properties of p230 Bcr-Abl and compare it with the p210 and p185 variants.

## Materials and methods

### P230 Bcr-Abl cDNA

An overlapping polymerase chain reaction (PCR) approach using a 3' Bcr primer, incorporating the known p230  $\mu$  breakpoint, was used to generate a 1013-base pair (bp) KpnI/HindIII Bcr-Abl cDNA fragment, spanning the  $\mu$  breakpoint region.<sup>19</sup> The primers (5'-GGAGCAGCAGAAGAGCTGTT-3') and (5'-GTCAGATGCTACTGGCCGCTGAAGGGCTTTGACGTCGAAGGCTGCC-3') were used with the EcoRI fragment of pGEM4[0]cBcr(3'UT) as the substrate<sup>23,24</sup> and primers (5'-AAGCCCTTCAGCGCCAG-3') and (5'-CTGCTCTCACTCTCAGCAC-3') were used with the EcoRI fragment of pGEM4cAbl1b as substrate.<sup>23,24</sup> These fragments were annealed and PCR-amplified using (5'-GGAGCAGCAGAAGAGCTGTT-3') and (5'-CTGCTCTCACTCTCAGCAC-3'). This fragment was sequenced in both directions to ensure that it contained the correct  $\mu$  Bcr-Abl breakpoint. The amplified fragment was then digested with KpnI and HindIII and ligated with the EcoRI/KpnI cAbl and KpnI/EcoRI Bcr fragments into the pSR $\alpha$ MSVtkneo retroviral vector. The generation of pSR $\alpha$ MSVtkneo-p185Bcr-Abl and pSR $\alpha$ MSVtkneo-p210Bcr-Abl has been previously described.<sup>24</sup>

### Cell culture

32D and DAGM cells were grown in Roswell Park Memorial Institute medium (RPMI) containing 10% fetal bovine serum (FBS), 10% WEHI-3B conditioned media (WEHI CM) as the source of IL3, and penicillin/streptomycin (P/S) antibiotics. Mouse bone marrow mononuclear cells (MNCs) were isolated from the femurs of twelve 3- to 4-week-old male Balb/C mice by density gradient through HISTOPAQUE-1077 (Sigma, St Louis, MO). Murine bone marrow MNCs were maintained in Iscove's modified Dulbecco medium (IMDM) with 15% FCS, 0.1% bovine serum albumin (BSA), 25  $\mu$ mol/L  $\beta$ -mercaptoethanol, and P/S with or without the following cytokines: 100 IU/mL recombinant murine IL3, 100 IU/mL recombinant murine IL6, and 1000 IU/mL (100 ng/mL) recombinant murine stem cell factor (SCF) (all obtained from InterGen, Purchase, NY). Cells were maintained at 37°C, 5% CO<sub>2</sub>.

### Gene transfer

For gene transfer into tissue culture cell lines, retroviruses were generated by transient transfection of 293T cells, as previously described.<sup>25</sup> Briefly, the various Bcr-Abl constructs were cotransfected with rsvv2 onto 293T fibroblasts<sup>26</sup> by calcium phosphate transfection in the presence of 25  $\mu$ mol/L chloroquine. Two days after transfection, Bcr-Abl-containing and control retroviral supernatants were collected and incubated with 1 million 32D, FL5-12, or DAGM cells in 10% WEHI-CM and 4  $\mu$ g/mL polybrene in a volume of 1 mL. Infections were performed at 37°C and 5% CO<sub>2</sub> for 3 to 6 hours. Cells were pelleted and resuspended in growth medium. Two days after infection, cells were selected by IL3 deprivation or resistance to G418

(0.3 to 0.7 mg/mL). IL3 deprivation was performed by washing the cells twice with RPMI and resuspending them in RPMI containing 10% FBS and P/S.

For gene transfer into primary mouse bone MNCs, p185 Bcr-Abl, p210 Bcr-Abl, and p230 Bcr-Abl were subcloned into the bicistronic retroviral vector MIGR1.<sup>27</sup> Retroviruses generated by transient transfection in 293T cells were used to infect GP + E-86 fibroblasts.<sup>26</sup> Three rounds of retroviral infection were performed over 2 days, after which these cells were trypsinized and selected for green fluorescent protein (GFP) expression using fluorescence activating cell sorting (FACS) on a Becton Dickinson (San Jose, CA) FACSTAR-PLUS with excitation at 488 nm. Bcr-Abl and GFP-coexpressing GP+E-86 retroviral producer lines were expanded in DMEM, 10% FBS, and P/S. These retroviral producer lines were resorted every 60 days to maintain a population that contained greater than 90% GFP-positive cells. Murine bone marrow MNCs were isolated by HISTOPAQUE-1077 (Sigma) gradient and between 2 and 5 million cells were plated onto confluent GP+E-86 producer lines. Immediately before gene transfer, GP + E-86 producer lines were gamma-irradiated at 1250 rads to prevent subsequent contamination of bone marrow cultures. Infections were carried out for 48 hours in the presence of 100 IU/mL recombinant murine IL3, 100 IU/mL recombinant murine IL6, and 1000 IU/mL (100 ng/mL) recombinant murine stem cell factor with 4  $\mu$ g/mL polybrene. The efficiency of gene transfer into primary mouse bone marrow cells between the various Bcr-Abl producer lines varied with the size of the construct, with transfer efficiency for p185 Bcr-Abl being generally 2 to 3 times greater than p230 Bcr-Abl, and p210 Bcr-Abl transfer efficiency generally between that of p185 and p230. Cocultivation with  $\gamma$ -irradiated GP + E-86 producer lines consistently improved gene transfer efficiency into primary murine stem cells by 25- to 50-fold, compared with infection with retroviral supernatants alone. Two days after retroviral infection, designated as "day zero," the murine bone marrow MNCs were used for experimentation. For in vitro differentiation experiments in the presence of autologous stroma, day zero murine bone marrow MNCs were plated directly after retroviral-mediated gene transfer and grown in the presence of 100 IU/mL recombinant murine IL3, 100 IU/mL recombinant murine IL6, and 1000 IU/mL (100 ng/mL) recombinant murine stem cell factor. We found that cocultivation, followed by plating in the presence of cytokines, reproducibly resulted in the formation of an adherent, confluent stromal layer and a nonadherent hematopoietic layer. Fresh growth media with cytokines was added every 2 days. The nonadherent cells were not passaged into new flasks to maintain contact with the adherent stromal compartment, which we found to be resistant to trypsinization. For in vitro experiments in the absence of autologous stroma, day zero GFP-expressing murine bone marrow MNCs were selected by FACS before plating either in the presence or absence of cytokines. We found that both control and GFP-containing cultures plated after GFP selection developed few, if any, adherent cells.

### Western blotting

Primary mouse bone marrow cells were lysed at a concentration of  $5 \times 10^7$  cells/mL directly in boiling  $2 \times$  sample buffer. Lysates were centrifuged at 100 000g for 25 minutes at 4°C and supernatants equivalent to  $1 \times 10^7$  cells were loaded onto SDS polyacrylamide gels for analysis. After gel electrophoresis, proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) and blotted with antibodies to Abl (8E9, Pharmingen, San Diego, CA) or phosphotyrosine (4G10, Upstate Biotechnologies, Lake Placid, NY) in 2% nonfat dry milk in TBS, followed by enhanced chemiluminescent detection (Amersham, Piscataway, NJ).

### Immunophenotyping

Cultures of Bcr-Abl and GFP-expressing or control GFP-expressing bone marrow cells were resuspended in phosphate-buffered saline (PBS) and 1% FBS. Cells were incubated with anti-CD16/CD32 ("Fc Block," Pharmingen) at 1.25  $\mu$ g/mL for 15 minutes on ice, followed by incubation with biotin-conjugated primary antibodies at 3.3  $\mu$ g/mL for 30 minutes on ice. Cells were washed 3 times with PBS and 1% FBS, followed by incubation with a combination of streptavidin-cy-chrome (Pharmingen) at 1.3  $\mu$ g/mL



and phycoerythrin (PE)-conjugated primary antibodies at 3.3  $\mu\text{g/mL}$  for 15 minutes on ice. Cells were washed twice with PBS and 1% FBS and analyzed on a Becton Dickinson FACStarPLUS, equipped with a 488 nm argon laser and tunable dye laser. For some analyses, dead cells were excluded by staining with 7AAD, in which case streptavidin-allophycocyan (Pharmingen) was used as the secondary reagent. Primary biotin and PE-conjugated antibodies used for this study were purchased from Pharmingen: B220 (clone RA3-6B2), Thy 1.2 (clone 53-2.1), Gr-1 (clone RB6-8C5), Mac-1 (clone M1/70), CD 43 (Ly-48), cKit (clone 2B8), CD 34 (clone RAM 34), CD9 (clone KMC8), and Sca1 (Ly-6A/E). FACS analysis was performed until 10 000 GFP positive events were acquired.

#### Cell cycle analysis

Cultures of Bcr-Abl and GFP-expressing or control GFP-expressing bone marrow cells were incubated with 10  $\mu\text{mol/L}$  bromodeoxyuridine (BrdU) for 30 minutes in IMDM with 15% FCS, 0.1% BSA, 25  $\mu\text{mol/L}$   $\beta$ -mercaptoethanol and P/S with or without cytokines at 37°C, and 5%  $\text{CO}_2$ . One million cells were then washed twice with PBS and 1% FBS and fixed with 5 mL 70% ethanol for 30 minutes on ice. This successfully extracted 100% of the GFP from the cells in control experiments. The cells were then stained with FITC-conjugated anti-BrdU antibody and propidium iodide as per the manufacturer's instructions (Becton Dickinson) and analyzed by flow cytometry on a Becton Dickinson FACStarPLUS with laser excitation at 488 nm.

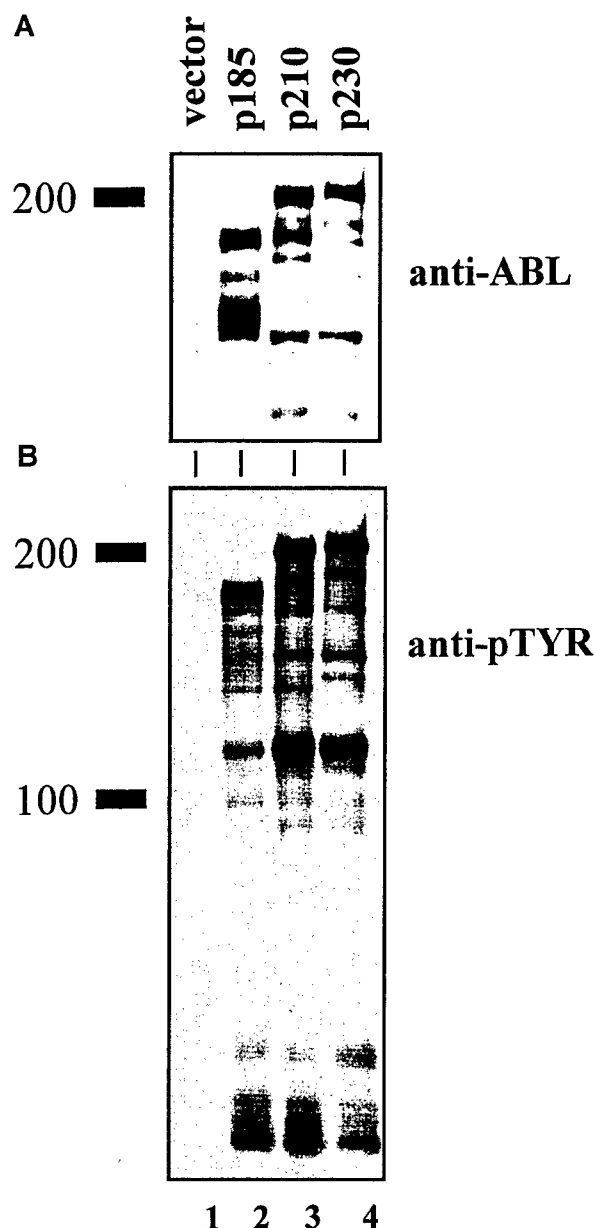
#### Tumor challenge

Cells were washed twice in RPMI only and resuspended at  $1 \times 10^7$  cells/mL RPMI containing 0.1% FBS. A total of  $1 \times 10^6$  cells (100  $\mu\text{L}$ ) were injected subcutaneously into the right flanks of 7- to 8-week-old male SCID mice (strain CB17SC-M, Taconic Farms, Germantown, NY).

## Results

**Cloning and expression of p230 Bcr-Abl.** We used an overlapping PCR technique with *Bcr* and *Abl* cDNA templates to create a *Bcr-Abl* chimeric cDNA that contained the unique p230  $\mu$  breakpoint.<sup>1,18,19</sup> After retroviral-mediated gene transfer of the various leukemia-associated Bcr-Abl isoforms into 32D cells, Western blot analysis with an anti-Abl antibody demonstrated expression of proteins of the expected sizes (Figure 1A). Western blotting with an antibody that recognizes the PH domain of Bcr showed that this domain is present in p210 and p230 Bcr-Abl, but not in p185 Bcr-Abl (data not shown). The tyrosine kinase activity of the Bcr-Abl chimeric oncoprotein is critical to its transforming ability.<sup>3,28</sup> 32D cells expressing the various leukemia-associated Bcr-Abl isoforms contained roughly equivalent levels of tyrosine-phosphorylated Bcr-Abl, along with additional phosphoproteins (Figure 1B). This finding indicates that p230 Bcr-Abl is kinase active, and its tyrosine kinase activity is similar to p210 and p185 Bcr-Abl oncoproteins in vivo.

**p230 Bcr-Abl renders 32D cells factor independent and activates similar signaling pathways as p185 and p210 Bcr-Abl in these cells.** Expression of p185 and p210 Bcr-Abl in 32D cells leads to growth factor independence and inhibition of apoptosis.<sup>3</sup> The ability of p230 Bcr-Abl to cause growth factor independence of 32D cells was compared with the p210 and p185 Bcr-Abl variants. After IL3 withdrawal, p230 cells grew at a rate equivalent to p210- and p185-expressing cells, whereas control cells underwent apoptosis (data not shown). p230 as well as p210- and p185-expressing 32D cells were resistant to the differentiating effects of GCSF, whereas control cells stopped cycling and differentiated into granulocytes (data not shown). Furthermore, p230 Bcr-Abl-expressing 32D cells were as potent as p185 and



**Figure 1.** Expression of p230 Bcr-Abl in 32D mouse myeloid cells leads to tyrosine phosphorylation of cellular proteins. Lysates of 32D cells expressing empty vector (lane 1), p185 (lane 2), p210 (lane 3), and p230 Bcr-Abl (lane 4) were analyzed by Western blotting with antibodies against (A) Abl (8E9, Pharmingen) or (B) phosphotyrosine (4G10, UBI).

p210 Bcr-Abl-expressing 32D cells in their ability to form tumors in SCID mice (Table 1). Thus, the biologic effects of the p230 Bcr-Abl isoform were indistinguishable from those of the more aggressive p210 and p185 isoforms of Bcr-Abl after expression of the proteins in 32D cells. Consistent with these findings, analysis of

**Table 1.** Tumorigenicity of 32D cells expressing distinct Bcr-Abl proteins

Bcr-Abl Form	Incidence*	Latency† (d)	Tumor Mass‡ (g)
32D/vector	0/4	21	NA
32D/p230	4/4	21	$1.3 \pm 0.3$
32D/p210	4/4	21	$3.4 \pm 0.3$
32D/p185	4/4	21	$1.5 \pm 0.9$

\*Number of mice with tumors/total number of mice injected.

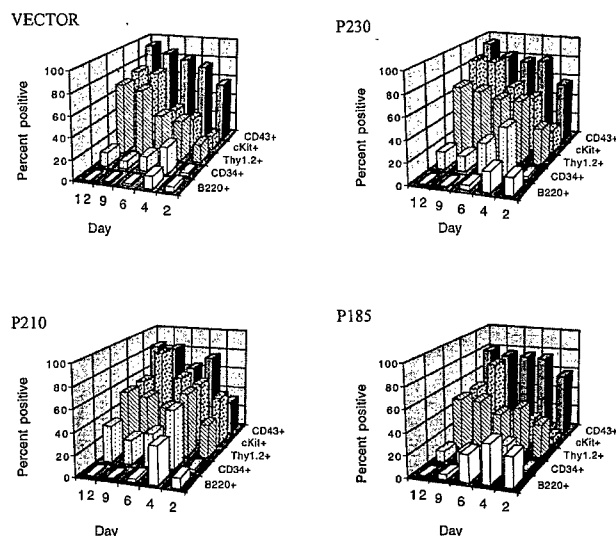
†Time in days when tumors were first observed.

‡Mean mass of tumors  $\pm$  1 SD.

the signaling properties of p230 Bcr-Abl in 32D cells revealed that p230 activated Ras, Erk, and Jnk to similar levels as those induced by p185 and p210 Bcr-Abl in these cells<sup>3,6,29</sup> (data not shown). Similar results were obtained in FL5-12 and DAGM cells expressing the 3 Bcr-Abl isoforms (data not shown). This data indicates that in lineage-restricted hematopoietic cell lines, the p185, p210, and p230 forms of Bcr-Abl activate Ras, Erk, and Jnk to similar extent, and elicit similar survival, proliferative, and tumorigenic effects.

**Effect of expression of distinct Bcr-Abl proteins on the differentiation of primary mouse bone marrow cultures expanded in the presence of exogenous cytokines and autologous stroma.** The effects of p230 Bcr-Abl expression were next studied on primary bone marrow cells, the natural target of Bcr-Abl related leukemias. We used a bicistronic retroviral vector to coexpress the leukemia-associated Bcr-Abl cDNAs with the gene for enhanced green fluorescent protein (GFP) as a selectable marker.<sup>27</sup> After retroviral-mediated gene transfer in primary bone marrow cells and selection for GFP, we observed equivalent expression of the 3 forms of Bcr-Abl (Figure 2A). Furthermore, the levels of in vivo phosphotyrosine were similar for cell lysates expressing the p185, p210, and p230 forms of Bcr-Abl (Figure 2B).

We developed hematopoietic culture conditions that would allow us to compare the ability of the 3 Bcr-Abl proteins to drive the differentiation of lymphoid versus myeloid lineages in vitro. Previous work has shown that both p185 and p210 Bcr-Abl drive lymphoid expansion of primary mouse bone marrow cells even under conditions that favor myeloid expansion.<sup>30</sup> We assayed the effects of the various leukemia-related Bcr-Abl tyrosine kinases on primary mouse bone marrow cells under 3 different conditions: (1) expansion in the presence of exogenous cytokines and autologous



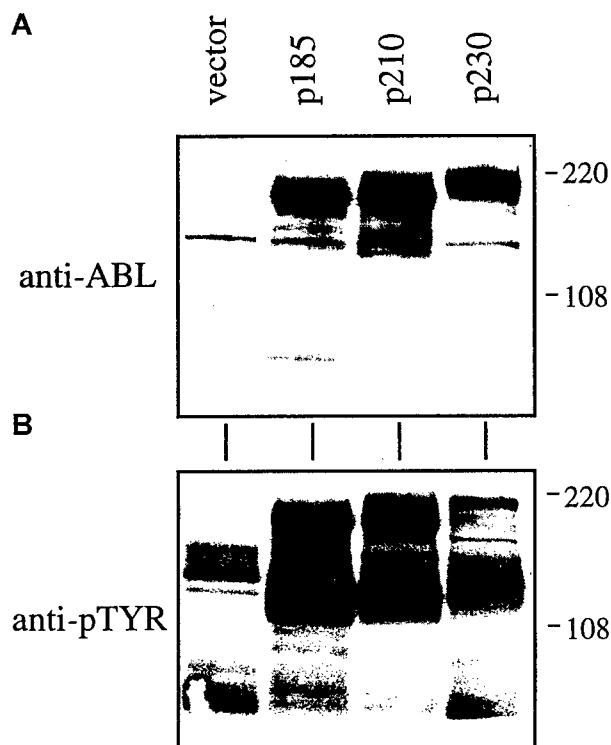
**Figure 3. Exogenous hematopoietic growth factors block the differentiation-inducing effects of the p185, p210, and p230 Bcr-Abl proteins in primary bone marrow cells.** After retroviral-mediated gene transfer and expansion in the presence of cytokines and autologous stroma, GFP-positive cells were analyzed for the expression of surface markers associated with B cells (B220), and stem cells (CD34, Thy1.2, cKit, and CD 43) at the indicated times after infection.

stroma; (2) expansion in the presence of exogenous cytokines alone; and (3) expansion without exogenous cytokines or autologous stroma.

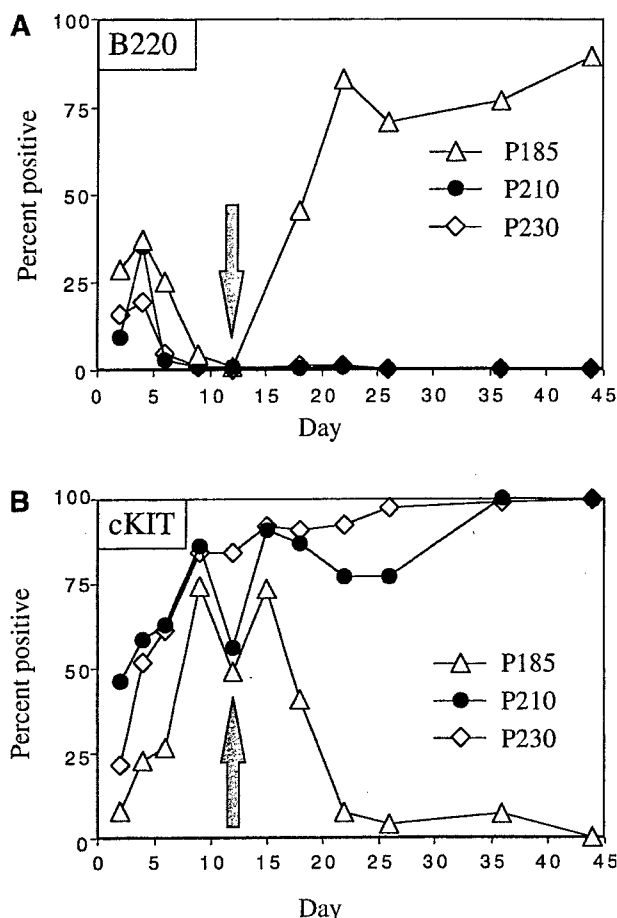
We first examined whether the various Bcr-Abl variants could overcome the differentiation-inhibitory effects of exogenous cytokines in the presence of autologous stroma.<sup>31</sup> We found that, when primary mouse bone marrow cells expressing the 3 different Bcr-Abl kinases and control bone marrow cells were expanded in the presence of cytokines and autologous stroma, outgrowth of B220 positive pre-B cells was inhibited up to 12 days in culture (Figure 3). This effect was dependent on the presence of autologous stroma and on the duration of cytokine exposure. p185 Bcr-Abl expressing cultures contained slightly more B220 positive cells in the first 6 days of the culture compared with p210, p230, and control bone marrow cells (Figure 3 and Figure 4A). However, by day 12, all cultures contained less than 1% B220 positive cells under these conditions. The loss of B220 expression in the bone marrow cultures was accompanied by the expansion of cKit positive cells, such that after 9 days of growth in the presence of cytokines and stroma, more than 70% of control and Bcr-Abl expressing bone marrow cells were cKit positive (Figure 3).

To determine whether p230 Bcr-Abl expression causes cytokine independence in primary cells, we tested the ability of day 12 bone marrow cultures expressing p230 Bcr-Abl to survive in the absence of cytokine and stromal support. Further, we compared the effects of p230 to those of p185 and p210 Bcr-Abl in this assay. We found that p185 Bcr-Abl-expressing primary bone marrow cultures expanded in the presence of cytokines and stroma were able to survive after cytokine and stromal withdrawal, resulting in the expansion of B220-positive pre-B cells (Figure 4A). These cells coexpressed CD24 and CD43 and were BP1 low (Figure 5B). Histologically, these cytokine and stroma-independent p185 Bcr-Abl-expressing bone marrow cells were small and contained few cytoplasmic granules (Figure 5A). Thus, these cells likely represent an immature B-cell progenitor.

The response of p210 and p230 Bcr-Abl-expressing mouse bone marrow cells to cytokine and stromal withdrawal was markedly different from that of p185 Bcr-Abl-expressing bone



**Figure 2. Expression of P230 Bcr-Abl in primary mouse bone marrow cells leads to tyrosine phosphorylation of cellular proteins.** Whole cell lysates were prepared from cytokine and serum-starved primary mouse bone marrow cells by boiling in 2 × sample buffer. Lysates were analyzed by SDS-PAGE and Western blotting with antibodies against (A) Abl or (B) phosphotyrosine.



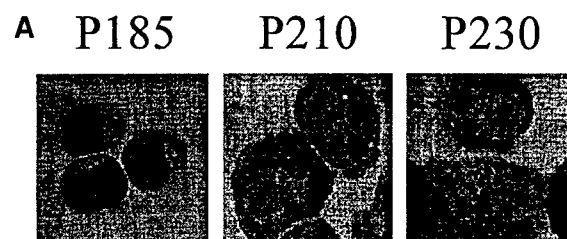
**Figure 4.** The presence of cytokines and autologous stroma inhibits the outgrowth of cytokine-independent p210- and p230-expressing pre-B cells. Two days after retroviral-mediated gene transfer, mouse bone marrow cells were expanded in the presence of autologous stroma and cytokines. After 12 days, nonadherent cells were replated in the absence of cytokines (indicated by the arrows). Cells were serially analyzed for the expression of B220 (A) and cKit (B).

marrow cells. Whereas cytokine and stromal withdrawal led to the expansion of cells that were cKit negative in p185 Bcr-Abl bone marrow cultures, p210 and p230 Bcr-Abl-expressing bone marrow cells retained cKit expression even after cytokine and stromal withdrawal (Figure 4B and Figure 5B). These cytokine- and stromal-independent p210 and p230 Bcr-Abl-expressing cells coexpressed CD9 and CD34, were positive for cKit (CD 117) (Figure 5B), were larger than the pre-B cells generated from p185-expressing cultures, and contained abundant azurophilic and basophilic granules (Figure 5A). All of these properties are consistent with differentiation into cells of the myeloid or monocyte lineages.<sup>32-38</sup> Thus, under the conditions used in this experiment, p185 Bcr-Abl exhibited a propensity to drive the expansion of cytokine-independent lymphoid progenitors, whereas p210 and p230 Bcr-Abl generated cytokine-independent cells of the myeloid/monocyte lineage.

**Effect of expression of distinct Bcr-Abl proteins on the differentiation, proliferation, and tumorigenicity of primary mouse bone marrow cultures expanded in the presence of exogenous cytokines without stroma.** To test the effect of the different Bcr-Abl chimeric proteins on the differentiation of primary mouse bone marrow cultures in the absence of autologous stroma, primary bone marrow cells were transduced with the Bcr-Abl- and GFP-coexpressing retroviruses, selected for GFP expression, and expanded in the presence of cytokines without stroma. After 10

days of growth in the presence of cytokines without autologous stroma, primary bone marrow cultures transduced with Bcr-Abl retroviruses encoding p185, p210, and p230 were either deprived of cytokines or injected subcutaneously into SCID mice. As shown in Figure 6, outgrowths of cytokine-independent cells immunophenotypically consistent with pre-B cells were obtained with all 3 Bcr-Abl isoforms from cultures that were initially predominantly B220 negative. The outgrowth of pre-B cells differed markedly among the various Bcr-Abl-expressing cultures. p185 and p210 Bcr-Abl-expressing mouse bone marrow cultures expanded with cytokines alone rapidly evolved into pre-B cells after cytokine withdrawal, whereas pre-B-cell development of p230 Bcr-Abl-expressing cells was delayed (data not shown).

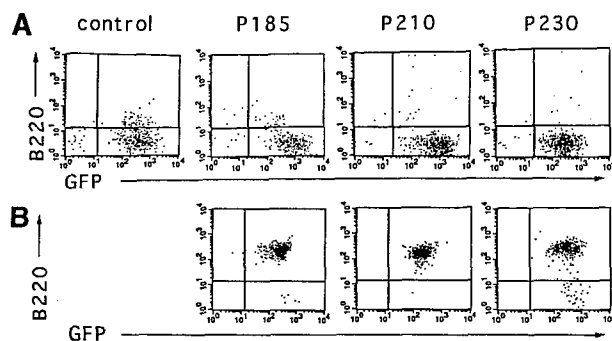
The effect of expression of the various Bcr-Abl tyrosine kinases on the growth of primary mouse bone marrow cells expanded with cytokines and without stroma was assessed by S-phase labeling with bromodeoxyuridine. In the presence of exogenous cytokines and without stroma, a larger percentage of p185 and p210 Bcr-Abl-expressing primary mouse bone marrow cells were in S-phase compared with p230 Bcr-Abl-expressing and control primary mouse bone marrow cells (Figure 7, upper panel).



**B Percent positive**

Antibody	P185	P210	P230
cKit	0.2	99.9	99.5
CD9	0.3	99.5	99.8
CD34	0.1	77.8	90.2
Sca1	0.1	8.6	10.5
B220	86.3	0.1	0.2
CD24	99.6	1.2	1.0
BP1	10.5	0.1	0.1
CD43	86.7	99.9	99.8

**Figure 5.** p185-expressing primary mouse bone marrow cells expanded with cytokines and stroma differentiate into pre-B cells after cytokine withdrawal, whereas p210- and p230-expressing cells differentiate into myeloid/monocyte cells under the same conditions. Two days after retroviral-mediated gene transfer, primary mouse bone marrow cells were expanded in the presence of autologous stroma and cytokines. After 12 days, nonadherent cells were replated in the absence of cytokines. Thirty-two days after replating (44 days after retroviral-mediated gene transfer), GFP-positive cells were subjected to cytospin and Wright's stain (A) or flow cytometry (B) to detect expression of the indicated cell surface markers.



**Figure 6. Cytokine withdrawal from Bcr-Abl-expressing primary mouse bone marrow cells after 10 days of cytokine support in the absence of autologous stroma leads to the outgrowth of pre-B cells.** Two days after retroviral-mediated gene transfer, Bcr-Abl and control cells were selected for expression of GFP by FACS. These cells were expanded in the presence of cytokines without autologous stroma for 10 days and analyzed for the expression of GFP and B220 (A). Six days after cytokine withdrawal from Bcr-Abl-expressing cultures (16 days after selection for GFP-expressing cells), cells were again analyzed by flow cytometry for expression of GFP and B220 (B). Vector-expressing cells were not viable after cytokine withdrawal.

Twenty-four hours after cytokine withdrawal, there was a significant reduction in the percentage of p230 Bcr-Abl-expressing and control cells in S-phase (Figure 7, lower panel). In contrast, the percentage of cells in S-phase in the p210 and p185 Bcr-Abl-expressing primary mouse bone marrow cultures decreased only slightly after cytokine withdrawal (Figure 7, lower panel). Thus, in primary mouse bone marrow cultures expanded in the absence of autologous stroma, p230 Bcr-Abl-expressing cells require cytokines for optimal growth, whereas p185 and p210 Bcr-Abl-expressing cells do not.

Next, we tested the tumorigenic potential of primary mouse bone marrow cultures expressing the 3 Bcr-Abl kinases expanded for 10 days in the presence of cytokines without stroma. At this time point, all cultures expressing the 3 Bcr-Abl proteins were negative for B220 (Figure 6A). p185 Bcr-Abl-expressing murine hematopoietic cells expanded with cytokines alone were able to rapidly form tumors when injected subcutaneously into SCID mice (Table 2). These p185 Bcr-Abl-expressing tumors coexpressed B220 and CD24 and were negative for sIgM, again consistent with a pre-B-cell phenotype (data not shown). p210 and p230 Bcr-Abl-expressing murine hematopoietic cells expanded with cytokines

**Table 2. Tumorigenicity of mouse primary bone marrow cells expressing distinct Bcr-Abl proteins**

Bcr-Abl Form	Incidence*	Latency† (d)	Tumor Mass‡ (g)
p185 (No 85)§	5/5	21	4.5 ± 0.7
p185 (No 103)§	4/4	21	5.4 ± 0.3
p210 (No 102)	0/5	150	0
p210 (No 106)	0/4	130	0
p230 (No 83)	0/5	160	0
p230 (No 97)  ¶	0/8	105-127	0
p230 (No 105)	0/4	130	0

\*Number of mice with tumors/total number of mice injected.

†Time in days when tumors were first observed (p185) or time at which animals without tumors were killed (p210 and p230).

‡Mean mass of tumors ± 1 SD.

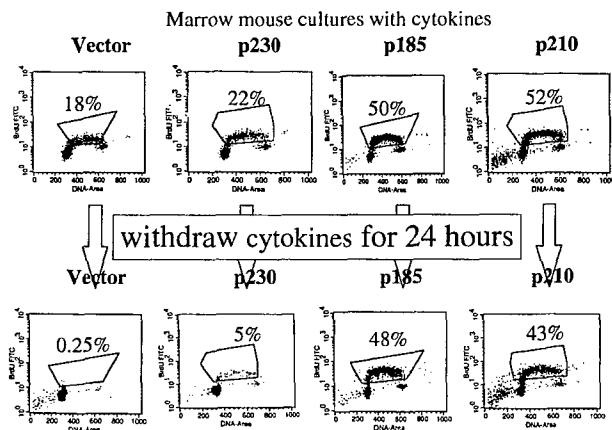
§All mice injected with p185 Bcr-Abl-expressing primary mouse bone marrow cells developed elevated white blood counts and splenomegaly with circulating GFP-positive lymphoid precursors consistent with the development of lymphoid leukemia.

||Indicates independently derived primary mouse bone marrow cultures.

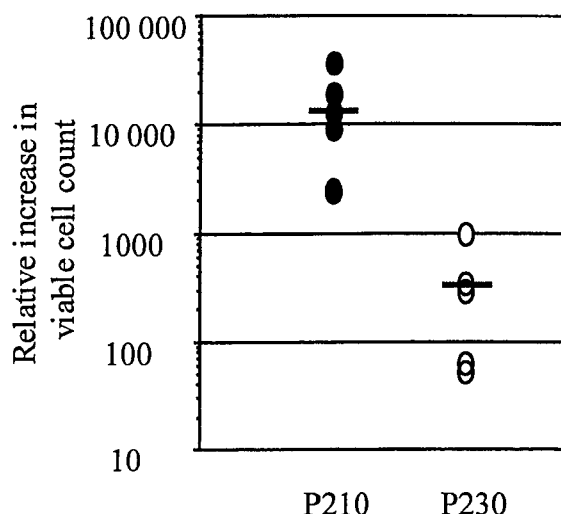
¶Five mice in this set died on days 105 to 128 but were free of tumor. The remaining 3 mice from this set were killed between days 121 and 127 and were also free from tumor; however, 2 of these mice had elevated white blood counts, splenomegaly, and polycythemia with circulating GFP-positive myeloid cells consistent with the development of a myeloid leukemia.

were unable to form tumors in SCID mice; however, 2 of 17 mice injected subcutaneously with p230-expressing bone marrow cells developed elevated white blood counts, splenomegaly, and polycythemia with circulating GFP-positive myeloid cells, consistent with the development of a CML-like syndrome. The decreased tumorigenic properties of p210 and p230 Bcr-Abl in this experiment were not due to lack of biologic activity. Transduction of bone marrow cells from 5-fluorouracil-treated mice with normalized retroviral supernatants expressing p230, p210, and p185 Bcr-Abl, revealed that, after injection of the transduced cells into the tail veins of lethally irradiated syngeneic recipient mice,<sup>27</sup> all mice developed a myeloproliferative disease (J. P. Miller and W. S. Pear, unpublished data). However, p230 Bcr-Abl caused a myeloproliferative disease with a significantly longer latency than that elicited by p185 and p210 Bcr-Abl (up to 27 weeks for p230 compared with 4 to 5 weeks for p185 and p210). Taken together, these data indicate that the proliferative and transforming properties of p230 are weaker than those of p185.

**p230 Bcr-Abl elicits a weaker proliferative response than p210 Bcr-Abl in the absence of cytokines and stroma.** To compare more directly the proliferative responses of p210 and p230 Bcr-Abl, we followed the growth of primary mouse bone marrow cultures expressing p210 or p230 2 days after retroviral-mediated gene transfer and selection for GFP expression in the absence of cytokines and autologous stroma. Ten days after GFP selection, p210 Bcr-Abl-expressing primary mouse bone marrow cells had expanded 13 000-fold, whereas p230 Bcr-Abl-expressing primary mouse bone marrow cells had barely expanded 300-fold, a 40-fold difference that was statistically significant ( $P < .05$ , Student *t* test) (Figure 8). After 10 days of growth without cytokines or stroma, p185, p210, and p230 Bcr-Abl-expressing cultures were all B220 positive. p185 Bcr-Abl cells grew at the same rate as p210 Bcr-Abl-expressing cells under these conditions, whereas murine bone marrow MNCs infected with the GFP-expressing retrovirus alone did not proliferate under these conditions (data not shown). Thus, although all leukemia-related Bcr-Abl isoforms can cause cytokine independence of primary murine bone marrow cells under a variety of conditions, they are markedly different in their abilities to drive the proliferation of these cells.



**Figure 7. p230-expressing primary mouse bone marrow cells require cytokines for optimal growth.** GFP-selected, control or Bcr-Abl-expressing primary mouse bone marrow cells were expanded for 10 days with cytokines in the absence of autologous stroma then split into duplicate cultures with (upper panels) and without (lower panels) cytokines. Twenty-four hours later, cultures were incubated in bromodeoxyuridine and analyzed for S-phase fraction.



**Figure 8. Relative increase in viable cell count of p210- and p230-expressing primary mouse bone marrow cultures expanded in the absence of either cytokines or autologous stroma.** Two days after gene transfer, p210 or p230 primary mouse bone marrow MNCs were selected for GFP expression by FACS. Data points represent the increase in total viable cell number relative to the number of GFP-positive cells recovered after FACS. Bars represent the mean of 6 independently derived p210 Bcr-Abl-expressing bone marrow cultures and 5 independently derived p230 Bcr-Abl-expressing bone marrow cultures.

## Discussion

The deregulation of normal hematopoiesis by chimeric oncogenes may result from dual effects on promoting cell growth and inhibiting cell differentiation, as well as enhancing cell survival. CML, considered the prototypical disease of the hematopoietic stem cell, is caused by the protein product of the p210 *Bcr-Abl* chimeric oncogene. The closely related p185 *Bcr-Abl* oncogene is the causative agent of a subset of ALL, whereas p230 *Bcr-Abl* has been associated with CNL, which has a clinical presentation that resembles the more typical p210 Bcr-Abl disease, but may have a more benign natural history.<sup>18</sup> Whether patients with p230 Bcr-Abl-related leukemia represent a bona fide stem cell disorder versus a myeloid lineage restricted disease remains to be established.<sup>39,40</sup> Furthermore, there is overlap between the disease spectrum associated with the different Bcr/Abl fusion proteins.<sup>1</sup> For example, p210 Bcr-Abl is associated with 40% of ALL, p185 Bcr-Abl with approximately 2% to 3% of CML,<sup>39,40</sup> and p230 Bcr-Abl has been described in patients with typical CML, some of whom have progressed to blast crisis.<sup>20-22</sup> It is not clear whether intrinsic differences in the activities of the 3 Bcr-Abl proteins account for their association with different disease phenotypes or whether the expression of each of the 3 Bcr-Abl forms is restricted to a distinct hematopoietic lineage, thus explaining their association with different leukemias. Data exist supporting the 2 possibilities.

It was recently shown by Li and colleagues<sup>41</sup> that p185 has greater in vitro tyrosine kinase activity than either p210 or p230 and that the proliferation rate of p185-expressing Ba/F3 lymphoid cells was greater than that of p210- and p230-expressing Ba/F3 cells in the absence of cytokines. In addition, mice reconstituted with p185 Bcr-Abl-expressing bone marrow cells that were not pretreated with 5-fluorouracil succumbed to lymphoid as well as other leukemias faster (4 weeks) than mice reconstituted with p210 and p230 Bcr-Abl-expressing marrow, which died by 10 and 11 weeks, respectively.<sup>41</sup> These findings are in agreement with our results that show that in primary mouse bone marrow cultures,

p230 was markedly impaired in its ability to generate cytokine-independent pre-B cells compared with p185. Indeed, p185 Bcr-Abl was very potent at driving lymphoid expansion, even under conditions that favored myeloid growth. In contrast, p210 and p230 generated cells of the myeloid/monocyte lineage under the same conditions. Thus, the association of p185 with primarily lymphoid diseases and p210 and p230 with primarily myeloid diseases may reflect the inherent ability of the various leukemia-associated Bcr-Abl proteins to promote lymphoid versus myeloid differentiation.

An alternative model that explains the association of the various Bcr-Abl proteins with leukemias of distinct phenotypes proposes that *Bcr* intron 1 breakpoints are much more common in lymphoid precursors, whereas breakpoints downstream in the *Bcr* gene occur more often in myeloid precursors. Indirect support for this model was provided by the finding by Li and colleagues<sup>41</sup> that all 3 forms of Bcr-Abl (p185, p210, and p230) induce an identical CML-like syndrome in mice receiving transduced bone marrow from 5-fluorouracil pretreated donors.<sup>41</sup> In contrast, under similar experimental conditions, we have found that p230 induced a myeloproliferative disease with a much longer latency compared with that induced by p185 and p210 Bcr-Abl (J. P. Miller and W. S. Pear, unpublished data). Future work is needed to explain the basis for the different results. In humans, there is a predominant association of each form of Bcr-Abl with a distinct spectrum of leukemias. However, the murine bone marrow transduction/transplantation model may not reveal marked differences in the disease phenotypes elicited by the 3 Bcr-Abl variants. Thus, alternative models may be required to dissect the molecular basis of the distinct pathologies linked to the expression of the various Bcr-Abl proteins. It has been proposed that the rarity of p190 (p185) Bcr-Abl CML is due to the restriction of m-Bcr breakpoints to progenitor cells already committed to lymphoid development.<sup>41</sup> However, this hypothesis fails to explain the frequent occurrence of Ph1<sup>+</sup> granulocytic and erythroid colonies in the marrow, and of Ph1<sup>+</sup> granulocytes in the blood of patients with Philadelphia chromosome positive ALL.<sup>42-44</sup> Our experiments do not specifically address the nature of the cell of origin of Bcr-Abl leukemias and future work will be necessary to directly address this issue.

Hematopoietic tissue culture cells have been useful in the elucidation of signal transduction pathways involved in Bcr-Abl leukemogenesis. These cell lines are typically restricted to either the myeloid or lymphoid lineage and may carry additional mutations as a consequence of adaptation to tissue culture. Using myeloid lineage-restricted hematopoietic cell lines, we have not found any differences in the biologic effects of p185, p210, and p230 Bcr-Abl. However, using lineage-unrestricted primary mouse bone marrow cells, we have shown that p230 Bcr-Abl is limited in its ability to drive the expansion of lymphoid progenitors, but drives the expansion of myeloid lineage cells.

The various Bcr-Abl oncoproteins share the same Abl tyrosine kinase sequences. Thus, the ability of Bcr-Abl to cause myeloid transformation of murine primary bone marrow stem cells may be modulated by the additional Bcr sequences found in the p210 and p230 forms of Bcr-Abl but not in p185 Bcr-Abl. Specifically, the Dbp-like and pleckstrin homology domains of p210, and the CalB and GAP<sup>rac</sup> domains of p230 may directly influence the ability of these proteins to transform various hematopoietic precursors by inhibition of lymphoid development, promotion of myeloid development, or both. The activity of the small GTP-binding protein Rac was found to be required for p210 Bcr-Abl-mediated transformation of 32D cells.<sup>45</sup> It is possible that the additional Bcr sequences

included within p230 Bcr-Abl, specifically the GAP<sup>rac</sup> domain, may function to partially abrogate the properties of activated p21 Rac in p230-expressing hematopoietic cells. Although only the first third of the Bcr GAP<sup>rac</sup> domain is included in p230 Bcr-Abl, it may function cooperatively with the CalB domain because these 2 domains are often found together.<sup>46</sup> Recently, additional support for a role of Bcr sequences in the induction of a myeloproliferative disease by Bcr-Abl in vivo has been provided through direct comparison of the leukemogenic activity of p210 Bcr-Abl with that of an activated Abl kinase that lacks Bcr sequences.<sup>47</sup> The latter induced only lymphoid malignancies in mice and did not stimulate the growth of myeloid colonies in vitro. In contrast, p210 Bcr-Abl efficiently induced a CML-like disease under the same conditions.<sup>47</sup>

The ability of Bcr-Abl to cause malignant transformation is a

function of multiple activities such as inhibition of apoptosis and altered cell adhesion and motility. We propose that the ability of Bcr-Abl proteins to direct distinct hematopoietic differentiation pathways is determined by the specific Bcr sequences that are included in the chimeric oncoproteins. The cell culture conditions described here should provide an opportunity to dissect the role of specific Bcr sequences in generating the diverse spectrum of leukemias associated with expression of the various Bcr-Abl tyrosine kinases.

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# Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway

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Oncogenic forms of the Abl and Src tyrosine kinases trigger the destruction of the Abi proteins, a family of Abl-interacting proteins that antagonize the oncogenic potential of Abl after overexpression in fibroblasts. The destruction of the Abi proteins requires tyrosine kinase activity and is dependent on the ubiquitin-proteasome pathway. We show that degradation of the Abi proteins occurs through a Ras-independent pathway. Significantly, expression of the Abi proteins is lost in cell lines and bone marrow cells isolated from patients with aggressive Bcr-Abl-positive leukemias. These findings suggest that loss of Abi proteins may be a component in the progression of Bcr-Abl-positive leukemias and identify a novel pathway linking activated nonreceptor protein tyrosine kinases to the destruction of specific target proteins through the ubiquitin-proteasome pathway.

[Key Words: Abi; Bcr-Abl; v-Src; ubiquitin-dependent proteolysis; Ph<sup>1</sup>-positive leukemia]

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Ubiquitin-dependent proteolysis is a critical component of diverse biological processes including cell cycle progression, the immune response, embryonic development, protein transport, and apoptosis (for review, see Deshaies 1995; Hochstrasser 1995; King et al. 1996; Varshavsky 1997). Here we show that ubiquitin-dependent proteolysis may also play a role in oncogenesis by activated nonreceptor tyrosine kinases.

Oncogenic forms of the Abl tyrosine kinase are linked to the development of human, murine, and feline leukemias (Bergold et al. 1987; Rosenberg and Witte 1988; Laneuville 1995; Gotoh and Broxmeyer 1997). Activation of cellular Abl (cAbl) oncogenic potential may occur as a consequence of chromosomal translocation events that generate chimeric fusion proteins such as Bcr-Abl and Tel-Abl (Gotoh and Broxmeyer 1997). The Bcr-Abl tyrosine kinases are produced by a reciprocal t(9;22)(q34;q11) chromosomal translocation that gives rise to the Philadelphia chromosome (Ph<sup>1</sup>). The translocation fuses varying amounts of the *Bcr* gene on chromosome 22 with sequences upstream of the second exon of the *c-Abl* gene on chromosome 9. Three different Bcr-Abl fusion proteins may be produced. The 210-kD form of Bcr-Abl (p210) is the causative agent of >95% of hu-

man chronic myelogenous leukemia (CML) cases (Clarkson et al. 1997; Gotoh and Broxmeyer 1997). The 185-kD Bcr-Abl protein (p185) is associated with a subset of acute lymphocytic leukemia (ALL) (Kurzrock et al. 1987). Recently, a rare 230-kD Bcr-Abl protein (p230) has been detected in patients with chronic neutrophilic leukemia (CNL) (Wada et al. 1995; Melo 1996). The p185 and p210 Bcr-Abl proteins have been shown to elicit ALL- and CML-like syndromes in mice, respectively, and to transform fibroblasts and hematopoietic cells in culture (Daley et al. 1990; Heisterkamp et al. 1990). Several mechanisms have been proposed to explain how Bcr-Abl transforms cells. Among these are increased resistance to apoptosis, enhanced proliferative capacity, defective adhesion, and increased motility of the Bcr-Abl-expressing cells (Clarkson et al. 1997; Cortez et al. 1997; Gotoh and Broxmeyer 1997; Salgia et al. 1997). The biological effects of Bcr-Abl require the constitutive tyrosine kinase activity of the chimeric protein. Multiple proteins have been identified as downstream targets of Bcr-Abl. Among these are proteins involved in the regulation of mitogenic and apoptotic pathways as well as cytoskeletal-associated proteins. Primarily, components of the Ras and phosphatidylinositol 3-kinase (PI3k) pathways are critical for Bcr-Abl-dependent transformation. Dominant interfering mutants of Grb-2, Ras, and c-Jun block Bcr-Abl-mediated transformation (Gishizky et al.

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1995; Raitano et al. 1995; Sawyers et al. 1995; Cortez et al. 1996). Grb-2, Ras, and c-Jun are components of the same signaling pathway, thereby emphasizing the essential role of this pathway in the transmission of the Bcr-Abl-transforming signal. Also, inhibition of PI3k and its downstream target, the Akt serine kinase, decreases transformation by Bcr-Abl (Skorski et al. 1995, 1997). In addition, interfering with the function of the transcription factors, c-Myc and NF- $\kappa$ B, abolishes transformation by Bcr-Abl (Sawyers et al. 1992; Reuther et al. 1998).

Less clear is the contribution of other downstream protein targets to Bcr-Abl-dependent transformation. Although the levels of the anti-apoptotic Bcl-2 mRNA and protein are elevated in some Bcr-Abl-expressing cells (Sanchez-Garcia and Grutz 1995), it is unclear whether Bcl-2 is up-regulated in all Bcr-Abl-expressing cells, and whether its up-regulation is necessary and sufficient for the anti-apoptotic activity of Bcr-Abl (Cortez et al. 1996). Likewise, it is not known what role the increased tyrosine phosphorylation of a number of cytoskeletal-associated proteins has on the altered adhesion and the overall transforming properties of Bcr-Abl-positive cells (Salgia et al. 1995).

Although numerous targets for the Abl kinases have been identified, only a few of these have been shown to be important in modulation of the Abl-transforming potential (Gotoh and Broxmeyer 1997). Recently, we and other researchers identified a family of Abl-interactor (Abi) proteins that bind specifically to both the SH3 and carboxy-terminal proline-rich sequences of Abl (Dai and Pendergast 1995; Shi et al. 1995). Two distinct, yet highly related genes, *abi-1* and *abi-2*, were identified and cloned. The corresponding protein products share overall 69% identity with the greatest homology observed in the amino-terminal homeobox-like domain, proline-rich sequences, and the carboxy-terminal SH3 domain. The Abi proteins are substrates of the Abl kinases. Significantly, Abi proteins antagonize the oncogenic activity of Abl in fibroblasts. Overexpression of Abi-1 potently suppresses the transforming activity of viral Abl (v-Abl) in NIH-3T3 fibroblasts (Shi et al. 1995). Furthermore, coexpression of a truncated form of Abi-2 with c-Abl activates the oncogenic potential of c-Abl (Dai and Pendergast 1995). These and other data (Wang et al. 1996; Biesova et al. 1997) suggest that the full-length Abi proteins may function as growth inhibitors in mammalian cells.

Inactivation of molecules that function as growth inhibitors/tumor suppressors is a common event in a large number of cancers (Cordon-Cardo 1995). As illustrated for p53 and pRb, the activity of the tumor suppressors may be abrogated by mutations of the corresponding DNAs or by the sequestration of the tumor suppressor proteins by specific viral or cellular proteins. Increasing evidence is accumulating that implicates selective proteolysis in the functional inactivation of tumor suppressor proteins (Deshaies 1995; Haupt et al. 1997; Kubbutat et al. 1997). In particular, ubiquitin-dependent proteolysis appears to play a role in this process. Protein degradation by the ubiquitin pathway involves the covalent attachment of multiple ubiquitin polypeptides to the

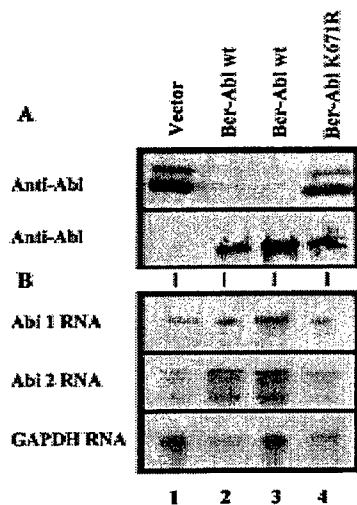
substrate protein, followed by the degradation of the polyubiquitinated substrate by the 26S proteasome, a large ATP-dependent multienzyme complex (Varshavsky 1997). Several proteins that function as growth inhibitors/tumor suppressors have been reported to be degraded through ubiquitin-dependent proteolysis. Among these are p53 and the cyclin-dependent kinase inhibitors Sic1p, Far1p, and p27 (Pagano et al. 1995; Feldman et al. 1997; Haupt et al. 1997; Henchoz et al. 1997; Kubbutat et al. 1997; Skowyra et al. 1997; Verma et al. 1997).

Here we show that oncogenic forms of the Abl and Src nonreceptor tyrosine kinases elicit the destruction of the Abi proteins by the ubiquitin-dependent proteasome machinery. The elimination of the Abi proteins by the oncogenic tyrosine kinases occurs through a novel Ras-independent pathway that is initiated by the constitutive tyrosine kinase activity of the oncoproteins. Significantly, the expression of Abi proteins is lost in cell lines and bone marrow cells from Ph<sup>1</sup>-positive leukemia patients. These findings suggest that loss of Abi proteins may play a role in oncogenesis and implicates ubiquitin-dependent proteolysis in tumor progression.

## Results

### *Bcr-Abl down-regulates Abi expression in hematopoietic cells*

To understand the role of Abi proteins in Abl-mediated transformation, we investigated the expression of Abi proteins in normal hematopoietic cells as well as in cells transformed by the oncogenic Bcr-Abl fusion protein. The *Bcr-Abl* gene was introduced into the pro-B BaF3 cell line and the multipotent myeloid progenitor 32D cell line. Both cell types are dependent on interleukin 3 (IL-3) for growth and survival. Expression of Bcr-Abl in BaF3 and 32D cells induces cellular transformation, confers cytokine-independent growth, and blocks apoptosis (Cortez et al. 1995, 1996, 1997). Abi proteins are expressed in BaF3 and 32D cells and migrate as a doublet of 60 and 65 kD (Fig. 1A, lane 1). Because of the strong homology between Abi 1 and Abi 2, the available anti-Abi antibodies cannot distinguish between the two proteins and therefore, we will refer to these protein bands as Abi. Surprisingly, little if any Abi protein could be detected in BaF3 cells transformed with p185 Bcr-Abl (Fig. 1A, lanes 2,3). Similar findings were obtained in 32D cells transformed by Bcr-Abl. These results indicate that the expression of the oncogenic Bcr-Abl tyrosine kinase down-regulates the expression of Abi proteins. Consistent with this notion, the expression of HA-tagged exogenous Abi 2 in 32D cells is also down-regulated by Bcr-Abl in the same manner as that of the endogenous Abi proteins (data not shown). The Bcr-Abl-mediated down-regulation of Abi expression requires Bcr-Abl tyrosine kinase activity, as the expression of the kinase-deficient mutant p185 K671R Bcr-Abl (Cortez et al. 1995) failed to down-regulate Abi expression (Fig. 1A, lane 4). To determine whether the loss of Abi expression in Bcr-Abl-transformed hematopoietic cells is caused by



**Figure 1.** The oncogenic Bcr-Abl tyrosine kinase down-regulates Abi protein expression in BaF3 cells. (A) Loss of Abi protein with p185Bcr-Abl expression. BaF3 cells ( $2 \times 10^6$ ) were infected (lanes 1,3,4) with the indicated retroviral expression vectors or stably transfected (lane 2) with the indicated plasmid. Abi proteins were detected by immunoblotting with polyclonal 5421 anti-Abi antibodies (top). Bcr-Abl (lanes 2,3) or Bcr-Abl (K671R) (lane 4) protein was detected by immunoblotting with monoclonal anti-Abl antibody (lower). (B) Bcr-Abl expression does not alter *abi 1* or *abi 2* mRNA levels. *abi 1*, *abi 2*, or *GAPDH* antisense RNA probes were protected against RNase digestion by BaF3 total RNA.  $^{35}$ S-labeled protected probes were electrophoresed on acrylamide/urea gels and detected by autoradiography.

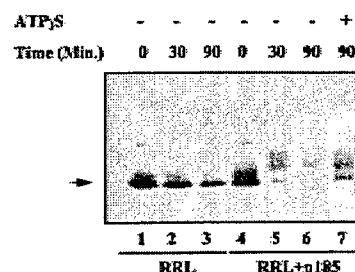
a decrease in the transcription of *abi* genes, Northern blot analysis and RNase protection assays were performed. BaF3 cells express message for both *abi 1* and *abi 2* (Fig. 1B, lane 1). No significant change in the *abi 1* and *abi 2* mRNA levels, however, was observed among cells expressing the vector control, wild-type p185 Bcr-Abl, or the p185 K671R Bcr-Abl mutant (Fig. 1B, cf. lanes 1-4). This suggests that Bcr-Abl down-regulates *abi* expression by a mechanism other than transcriptional regulation.

#### *Bcr-Abl* down-regulates Abi expression through the ubiquitin-proteasome pathway

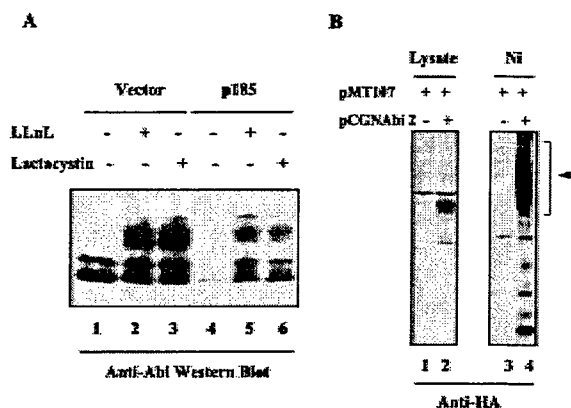
The oncogenic Bcr-Abl proteins down-regulate Abi expression without significantly reducing the level of *abi* transcripts. This suggests that the down-regulation may occur post-translationally. An increasing number of cellular processes have been shown to be critically dependent on the control of protein abundance catalyzed by the ubiquitin-dependent proteasome pathway (Deshaies 1995). Therefore, we tested whether the down-regulation of Abi expression by Bcr-Abl uses this pathway. First, we examined whether the down-regulation of Abi proteins by Bcr-Abl is caused by increased instability of the pro-

teins. To this end we synthesized  $^{35}$ S-labeled Abi 2 in vitro using a rabbit reticulocyte lysate (RRL) and tested its stability in the presence or absence of in vitro-translated Bcr-Abl. The RRL system has been used commonly as the source of active ubiquitinating enzymes and proteasome complexes (Nielsen et al. 1997; Pagano et al. 1997). In the absence of Bcr-Abl, Abi 2 was relatively stable. In contrast, in the presence of Bcr-Abl Abi 2 was degraded rapidly in the RRL (Fig. 2, cf. lanes 3 and 6). This suggests that Bcr-Abl increases Abi protein instability. Consistent with this, we found that addition of ATP $\gamma$ S, a nonhydrolyzable ATP analog that prevents degradation of ubiquitinated proteins by the proteasome but does not prevent their ubiquitination, blocked Abi 2 degradation in RRL system (Fig. 2, lane 7).

We then tested whether the down-regulation of Abi proteins by Bcr-Abl in hematopoietic cells is dependent on the ubiquitin-proteasome pathway. BaF3 cells transfected with either a control vector or an expression vector for p185 Bcr-Abl were treated with two specific inhibitors of the ubiquitin-proteasome machinery, LLnL (*N*-acetyl-L-leucyl-L-leucyl-L-norleucinal) and lactacystin (Aberle et al. 1997). As shown by Western blot analysis, LLnL and lactacystin inhibited the down-regulation of Abi expression in Bcr-Abl-expressing cells (Fig. 3A, lanes 5,6). Treatment of BaF3 cells expressing p185 Bcr-Abl with LLnL and lactacystin resulted in the accumulation of 60- and 65-kD Abi proteins, as well as immunoreactive Abi proteins with a slower mobility in SDS gels. The slower mobility bands may represent Abi proteins that are modified during the process of ubiquitin-mediated proteolysis. Interestingly, the Abi proteins, in particular the slower mobility forms, also accumulated to higher levels in the control BaF3 cells treated with LLnL and lactacystin (Fig. 3A, lanes 2,3). This suggests that Abi expression in normal cells may also be regulated, at least in part, by ubiquitin-mediated proteolysis. We then examined whether Abi proteins are targets for ubiquitination. A plasmid expressing an HA-tagged Abi 2 was transfected into Bosc 23 cells in the



**Figure 2.** In vitro degradation of Abi 2 is stimulated by p185Bcr-Abl and is ATP dependent. Abi 2 was synthesized in the presence of [ $^{35}$ S]methionine using a coupled transcription-translation kit (Promega). The labeled Abi 2 was incubated in a protein-degradation reaction mix with or without unlabeled p185Bcr-Abl, and with or without ATP $\gamma$ S, as indicated. Samples (equal volume) were removed at indicated time points and analyzed by SDS-PAGE and fluorography.



**Figure 3.** Bcr-Abl down-regulates Abi expression through an ubiquitin-dependent proteolysis pathway. (A) Proteasome-specific inhibitors LLnL and lactacystin inhibit Bcr-Abl-induced Abi down-regulation. BaF3 cells transfected by vector alone (lanes 1–3) or expression vector encoding p185 Bcr-Abl (lanes 4–6) were untreated (lanes 1, 4) or treated with 50  $\mu$ M LLnL (lane 2, 5) or 10  $\mu$ M lactacystin (lanes 3, 6) for 8 hr. Cells ( $2 \times 10^6$ ) were lysed in SDS sample buffer and subjected to Western blot analysis with 5421 anti-Abi antibodies. (B) Abi 2 is ubiquitinated in Bosc 23 cells. Bosc 23 cells were cotransfected with pMT107, a plasmid expressing His<sub>6</sub>-tagged ubiquitin, plus either pCGN control plasmid (lanes 1, 3) or pCGN-Abi 2 plasmid encoding HA-tagged human Abi 2 (lanes 2, 4). Total cell lysates (lanes 1, 2) or ubiquitin substrate conjugates that were affinity precipitated by Ni<sup>2+</sup> chelate chromatography (lanes 3, 4) were subjected to Western blot analysis with anti-HA monoclonal antibody. The position of ubiquitinated Abi 2 is indicated by an arrowhead.

presence of an expression plasmid encoding His<sub>6</sub>-tagged ubiquitin (Treier et al. 1994). The His<sub>6</sub>-tagged ubiquitinated proteins were purified by Ni-agarose chromatography (Treier et al. 1994; Aberle et al. 1997) and subjected to Western blot analysis with monoclonal antibody to HA to detect the HA-tagged Abi 2 protein. As shown in Figure 3B, Abi 2 is ubiquitinated.

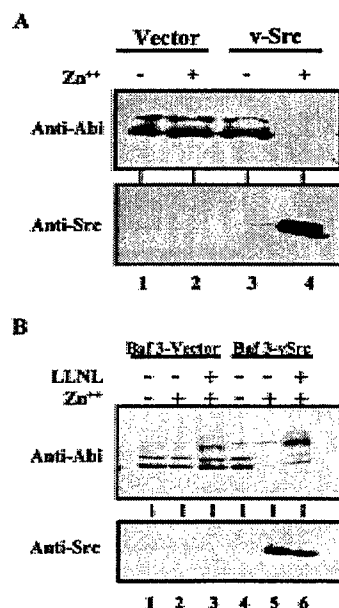
#### Oncogenic Src tyrosine kinase down-regulates Abi expression

We then wanted to test whether Abi proteins could also be down-regulated by expression of other oncogenic tyrosine kinases such as v-Src. A BaF3 cell line transfected with a zinc-inducible v-Src expression plasmid (Canman et al. 1995) was used in this experiment. The cells were treated with or without zinc for 8 hr to induce v-Src expression and cell lysates were subjected to Western blot analysis with either Abi (Fig. 4A, top) or v-Src (Fig. 4A, bottom) specific antibodies. Expression of v-Src was increased dramatically with the addition of zinc and the increased expression of v-Src correlated with a dramatic reduction of Abi expression (Fig. 4A, cf. lane 3 to lane 4). Thus, like Bcr-Abl, the oncogenic v-Src tyrosine kinase also down-regulates Abi expression in BaF3 cells. To determine whether the v-Src induced down-regulation of

Abi expression is mediated by the ubiquitin-proteasome degradation pathway, we examined the effect of LLnL on Abi expression in v-Src-expressing BaF3 cells. v-Src-transfected cells were incubated with zinc in the presence or absence of LLnL. Zinc induced expression of v-Src (Fig. 4B, lanes 5, 6), regardless of the presence or absence of LLnL. Zinc induced expression of v-Src (Fig. 4B, lanes 5, 6), regardless of the presence or absence of LLnL. Down-regulation of Abi expression in cells treated with LLnL was completely inhibited compared with cells without LLnL treatment (Fig. 4B, cf. lanes 5 and 6). This result demonstrates that v-Src down-regulates Abi expression through ubiquitin-mediated proteolysis.

#### Bcr-Abl-mediated down-regulation of Abi expression is Ras independent

Oncogenic Bcr-Abl proteins elicit cellular transformation through multiple signal transduction pathways (Gotoh and Broxmeyer 1997). Previously, we and other investigators have shown that Ras function is activated

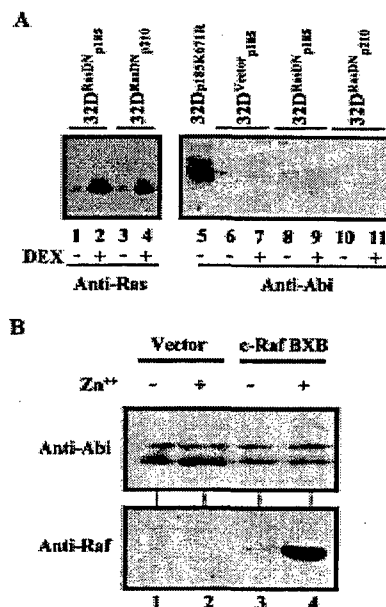


**Figure 4.** v-Src down-regulates Abi expression through an ubiquitin-dependent proteolysis pathway. (A) Oncogenic v-Src tyrosine kinase down-regulates Abi expression. BaF3 cells transfected by vector alone (lanes 1, 2), or zinc-inducible v-Src-expressing vector (lanes 3, 4) were treated with or without 75  $\mu$ M ZnCl<sub>2</sub> for 8 hr, as indicated. Total cell lysates ( $2 \times 10^6$ ) were subjected to Western blot analysis with either 5421 anti-Abi antibodies (top) or anti-v-Src antibodies (bottom). (B) Proteasome inhibitor LLnL inhibits v-Src-induced down-regulation of Abi expression. BaF3 cells transfected by vector alone (lanes 1–3) or zinc-inducible v-Src expression vector (lanes 4–6) were treated with (lanes 3, 6) or without (lanes 1, 2, 4, 5) 50  $\mu$ M LLnL for 1 hr. ZnCl<sub>2</sub> (75  $\mu$ M) was then added as indicated, and cells were incubated for an additional 7 hr. Cells ( $2 \times 10^6$ ) were lysed in SDS sample buffer and subjected to Western blot analysis with either 5421 anti-Abi (top) or anti-v-Src antibodies (bottom).

in Bcr-Abl-transformed cells and it is a necessary component for Bcr-Abl-mediated transformation (Pendegast et al. 1993; Puil et al. 1994; Cortez et al. 1995, 1996). Studies on photoreceptor cell differentiation of the *Drosophila* eye have shown that activation of the Ras/Map kinase-signaling cascade results in the ubiquitin-mediated degradation of Tramtrack (TTK) (Li et al. 1997; Tang et al. 1997), a transcriptional repressor of neuronal cell fates, as well as of the transcription factor YAN (Rebay and Rubin 1995), a general inhibitor of differentiation of many cell types in the *Drosophila* eye. Therefore, we examined whether the Bcr-Abl-mediated down-regulation of Abi expression is Ras dependent. We used p185 and p210 Bcr-Abl-transformed 32D cells that coexpress a dominant-negative form of Ras, Ras Asn 17 (Feig and Cooper 1988), under the control of a glucocorticoid responsive promoter. Previously, we have shown that the inducible expression of dominant-negative Ras Asn 17 blocks Bcr-Abl from activating Ras in these cells (Cortez et al. 1996). Cells were treated with dexamethasone for 24 hr to obtain high-level expression of dominant-negative Ras Asn 17. The expression of Bcr-Abl (data not shown), dominant-negative Ras (Fig. 5A, lanes 1-4), and Abi proteins (Fig. 5A, lanes 5-11) was evaluated by Western blot analysis. Despite overexpression of dominant-negative Ras Asn 17, the expression of Abi proteins in the dexamethasone-treated cells (Fig. 5A, lanes 9,11) is down-regulated to low levels similar to nontreated cells (Fig. 5A, lanes 8,10) or control cells that express Bcr-Abl alone (Fig. 5A, lanes 6,7). Because Raf is an immediate downstream component of Ras in the Ras/Map kinase signaling cascade, we tested whether the enforced expression of an activated Raf protein kinase would elicit the down-regulation of Abi expression in BaF3 cells. A BaF3 cell line that inducibly expresses an activated form of human c-Raf (c-Raf-BXB) (Canman et al. 1995) from a zinc-responsive promoter was grown in the presence or absence of zinc. Zinc induced the expression of c-Raf-BXB (Fig. 5B). Consistent with the findings in Figure 5A, the enforced expression of the activated Raf did not affect the expression of Abi protein (Fig. 5B, cf. lane 3 to lane 4). Taken together, our results demonstrate that down-regulation of Abi expression by oncogenic Bcr-Abl is Ras and Raf independent.

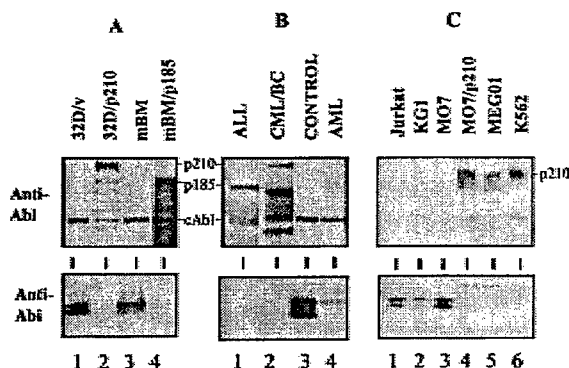
#### The expression of Abi proteins is lost in cell lines and bone marrow cells from Philadelphia chromosome-positive leukemia patients

The finding that Bcr-Abl down-regulates the expression of Abi proteins in BaF3 cells and 32D cells prompted us to test whether the Abi protein levels are also down-regulated as a consequence of Bcr-Abl expression in primary bone marrow cells, the natural target of the oncogenic Bcr-Abl tyrosine kinase. Mouse bone marrow cells were infected with Bcr-Abl and the expression of Abi proteins was examined by Western blotting (Fig. 6A, bottom). Consistent with the results observed in BaF3 and 32D cells (Fig. 6A, lanes 1,2), infection of bone marrow cells with Bcr-Abl retrovirus results in a loss of Abi ex-



**Figure 5.** Bcr-Abl-mediated down-regulation of Abi expression is independent of Ras signaling. (A) Expression of a dominant-negative Ras, Ras Asn 17, failed to block Bcr-Abl-mediated down-regulation of Abi expression. 32D cells expressing a control plasmid (32D<sup>Vector</sup>, lanes 6,7) or a plasmid inducibly expressing Asn 17 Ras (32D<sup>RasDN</sup>, lanes 1-4 and 8-11) were infected with retroviruses carrying either the p185Bcr-Abl (lanes 1,2, and 6-9) or p210Bcr-Abl (lanes 3,4,10,11) oncogenes. The cells were treated with or without 50 nM dexamethasone for 24 hr, as indicated, to induce the expression of Ras Asn 17. A control 32D cell line infected with a retrovirus encoding a kinase-deficient p185Bcr-Abl mutant was also included (lane 5). Cells ( $2 \times 10^6$ ) were lysed in SDS sample buffer and subjected to Western blot analysis with either anti-Ras antibody (pan Ras, Santa Cruz) (lanes 1-4) or 5421 anti-Abi antibody (lanes 5-11) as indicated. (B) Expression of an activated form of c-Raf does not down-regulate Abi expression. BaF3 cells transfected with either vector alone (lanes 1,2), or an inducible expression vector encoding activated c-Raf, c-Raf-BXB, were treated with or without 75  $\mu$ M ZnCl<sub>2</sub> for 8 hr as indicated. Cells ( $2 \times 10^6$ ) were lysed in SDS sample buffer and subjected to Western blot analysis using 5421 anti-Abi antibody (top) or anti-Raf antibody (Santa Cruz, bottom).

pression (Fig. 6A, cf. lanes 3 and 4, bottom) that correlates with the expression of Bcr-Abl protein (Fig. 6A, top). Then we tested whether the expression of Abi proteins is also reduced in bone marrow cells from patients with Ph<sup>1</sup>-positive human leukemias. The Abi proteins were shown to be expressed in bone marrow cells from either normal human samples or a Ph<sup>1</sup>-negative leukemia patients (Fig. 6B, lanes 3,4). In contrast, Abi protein expression was lost in bone marrow cells from a Ph<sup>1</sup>-positive patient with ALL or from a Ph<sup>1</sup>-positive patient with CML in the blast crisis phase of the disease (Fig. 6B, lanes 1,2). K562 and MEG01 are cell lines derived from patients with CML in the blast crisis phase of the dis-



**Figure 6.** Expression of Abi proteins is lost in Bcr-Abl-transformed primary bone marrow cells and cells from Ph<sup>1</sup>-positive leukemia patients. Western blots were performed to compare Abi immunoreactivity (*top*) with Abi protein expression (*bottom*) in murine cells, human leukemia samples, and human cell lines. (A) 32D cells were infected with retroviral constructs containing vector alone (32D/v, lane 1) or p210 Bcr-Abl (32D/p210, lane 2). Primary mouse bone marrow (mBM, lane 3) was infected with a retroviral construct containing p185 Bcr-Abl (mBM/p185, lane 4) and analyzed after 18 days of selection with G418. (B) Bone marrow was obtained from patients with Ph<sup>1</sup>-positive acute lymphoblastic leukemia (ALL, lane 1), or with Ph<sup>1</sup>-positive chronic myelogenous leukemia in blast crisis (CML/BC, lane 2), and was compared to normal bone marrow (control, lane 3) and bone marrow obtained from a patient with Ph<sup>1</sup>-negative acute myelogenous leukemia (AML, lane 4). (C) Ph<sup>1</sup>-negative human leukemia cell lines (Jurkat and KG1, lanes 1,2) and human myeloid cell line MO7 (lane 3) were compared to Ph<sup>1</sup>-positive cell lines (MEG01 and K562, lanes 5,6) or to a Ph<sup>1</sup>-negative cell line infected with retrovirus containing p210 Bcr/Abl (MO7/p210, lane 4).

ease. Therefore, we compared these cell lines with a human myeloid cell line (MO7e), a cell line from a Ph<sup>1</sup>-negative acute myelogenous leukemia patient (KG1), and a cell line from a T-cell leukemia patient (Jurkat), for the expression of Abi proteins. Examination of the mRNAs for *abi 1* and *abi 2* by reverse-transcriptase PCR revealed that they are present in all of these cell lines (data not shown). As shown in Figure 6C, expression of Abi proteins was undetectable in those cells from the Ph<sup>1</sup>-positive leukemia patients, whereas Abi proteins are present in the other cell lines (Fig. 6C, lanes 5,6). Consistently, the down-regulation of Abi expression correlates with the expression of oncogenic Bcr-Abl (Fig. 6C, upper). Taken together, these data suggest that loss of Abi protein expression may be a component in the progression of Bcr-Abl-positive leukemias.

## Discussion

We have identified a novel pathway downstream of the oncogenic Abl and Src nonreceptor tyrosine kinases that targets the destruction of the Abl-interacting Abi family of proteins through the ubiquitin-proteasome pathway. The down-regulation of the Abi proteins requires the tyrosine kinase activity of Abl, and it is independent of the

Ras-Raf pathway. Significantly, the degradation of the Abi proteins appears to be selective. Other molecules known to be degraded by the ubiquitin-dependent proteolysis pathway in response to extracellular signals or cell cycle progression, such as I $\kappa$ B $\alpha$  and the cyclin-dependent kinase inhibitor p27, are not affected by expression of oncogenic forms of Abl (Reuther et al. 1998; Z. Dai and A.M. Pendergast, unpubl.). It is likely, however, that additional proteins may be targeted for ubiquitin-dependent degradation after expression of the Abl and Src oncogenic tyrosine kinases.

It has become increasingly apparent that ubiquitin-dependent proteolysis of specific proteins is a highly regulated process. Linkage of ubiquitin to proteins that display a distinct degradation signal, results in the destruction of the ubiquitin-protein conjugate by the 26S proteasome (Varshavsky 1997). The conjugation of ubiquitin to the target protein involves a series of steps that begin with the formation of a thioester bond between ubiquitin and the ubiquitin-activating enzyme (E1). Ubiquitin is then transesterified to an ubiquitin-conjugating enzyme (UBC or E2) and subsequently transferred to the target protein, usually with the involvement of an ubiquitin protein ligase (E3). The latter is the component of the ubiquitin conjugation system that is involved in substrate recognition (Varshavsky 1997). Several ubiquitin-dependent degradation signals have been identified to date. Regulated destruction of target proteins is usually dependent on phosphorylation, interaction with specific proteins, or both. Most of the phosphorylation-regulated degradation signals identified to date are mediated by serine/threonine kinases (Rebay and Rubin 1995; Henchoz et al. 1997; Maniatis 1997; Verma et al. 1997). Although the tyrosine kinase activity of Bcr-Abl is absolutely required for Abi degradation, it is not clear at present whether direct tyrosine phosphorylation of the Abi proteins is critical for their proteolytic degradation. It is possible that the activated tyrosine kinases may induce Abi degradation through the phosphorylation of serine/threonine residues on Abi by protein kinases activated downstream of the oncogenic tyrosine kinases. Alternatively, the activated tyrosine kinases may induce the formation of a complex between Abi proteins and specific cellular proteins that target Abi for degradation. These two types of degradation signals are not mutually exclusive. Multiple serine, threonine, and tyrosine residues are found in the Abi proteins that may be phosphorylated by various protein kinases. Abi protein also contain sequences rich in proline, glutamic acid, serine, and threonine, designated as PEST, which have been found in many proteins that are targeted for ubiquitin-dependent degradation (Deshaies 1995; Rechsteiner and Rogers 1996). However, PEST sequences alone are not sufficient to identify those proteins that are targets of ubiquitin-dependent degradation (Varshavsky 1997). Extensive mutagenesis of the Abi proteins is necessary to identify those residues critical for their ubiquitin-dependent degradation. The availability of an *in vitro* degradation assay for the Abi proteins (Fig. 2) will facilitate the identification of the residues on Abi important for ubiquitin-de-

pendent degradation and will permit the isolation of the protein recognition complex that targets Abi for degradation by the proteasome.

The finding that Abi protein expression is lost in cells after expression of the transforming Bcr-Abl and v-Src tyrosine kinases, together with the discovery that Abi proteins are absent in cell lines and bone marrow cells isolated from patients with aggressive Bcr-Abl-positive leukemias, suggests that loss of Abi proteins by the ubiquitin-proteasome pathway may be a component in the progression of Bcr-Abl-positive leukemias and possibly other cancers. The irreversible nature of proteolysis makes this process uniquely suited for the elimination of growth inhibitory molecules during tumor progression. Indeed, several tumor-suppressor and growth-inhibitory proteins have been shown to be degraded by ubiquitin-dependent proteolysis. Among these is the p53 tumor-suppressor protein that is targeted for degradation by the human papilloma virus E6 oncoprotein (Scheffner et al. 1990) and the cellular Mdm-2 protein (Haupt et al. 1997; Kubbutat et al. 1997). Similarly, SHP-1, a protein tyrosine phosphatase that is implicated in receptor-mediated inhibitory signals, is targeted for ubiquitin-dependent degradation by an activated form of the Kit receptor tyrosine kinase (Piao et al. 1996). More recently, another link between tumor progression and increased proteasome-dependent degradation was provided by the finding that the cell cycle inhibitor p27 is targeted for ubiquitin-dependent degradation in aggressive colorectal carcinomas (Loda et al. 1997). These examples show that selective degradation of proteins that participate in the control of growth inhibitory pathways represents an alternative mechanism for their inactivation without the involvement of mutations or deletions in the corresponding genes.

Although our findings and those of other published reports are consistent with the hypothesis that Abi proteins function as growth inhibitors/tumor suppressors, an alternative role for these proteins, which cannot be ruled out at the present time, is that Abi proteins are downstream substrates of the oncogenic tyrosine kinases. The phosphorylated Abi proteins may transduce a signal from the oncogenic tyrosine kinases. Abi activation may be coupled to Abi destruction through the ubiquitin-proteasome pathway. The tightly coupled activation and the proteasome-dependent destruction of a protein has been documented for the p58 component of the yeast kinetochore Cbf 3 protein complex (Kaplan et al. 1997). The p58 protein is activated by phosphorylation as assayed by DNA-binding activity and subsequently it is degraded by the proteasome in a ubiquitin-dependent step. The phosphorylation and degradation of p58 are tightly coupled events that require the product of the *SKP1* gene p23 Skp1 (Kaplan et al. 1997). It has been proposed that p23 Skp1 functions as an adaptor that recruits a protein kinase by binding to both p58 and the unknown kinase. Also, p23 Skp1 is a component of the E3 ubiquitin ligase complex that targets p58 to the proteasome machinery. In this manner p58 is regulated positively by phosphorylation and regulated negatively

by ubiquitin-dependent proteolysis. Linked activation and negative regulation of cellular proteins in response to phosphorylation has also been reported in mammalian cells for the STAT 1 transcription factor (Kim and Maniatis 1996). Activated STAT 1 has been shown to be regulated negatively by the ubiquitin-proteasome pathway. Tyrosine phosphorylation of STAT 1, which is induced by treatment of cells with interferon- $\gamma$ , is required for its nuclear translocation and activation of transcription and it is also required for STAT 1 ubiquitination and subsequent degradation. A similar role for the phosphorylation of Abi proteins may exist in cells transformed by the activated Abl and Src tyrosine kinases.

The finding that Abi proteins are targeted for degradation by both oncogenic Abl and Src suggests that the activities of these two tyrosine kinases may be linked or alternatively, that they may regulate independently the function of specific common target proteins such as Abi. The data also raise the possibility that Abi proteins may be targeted for degradation in other human cancers where the Src family of nonreceptor tyrosine kinases are activated constitutively. Future work will be geared toward elucidating the cellular components of this novel pathway and the identification of additional targets of ubiquitin-dependent degradation triggered by the activity of oncogenic protein tyrosine kinases.

## Materials and methods

### Cell culture and retroviral infection

BaF3 cells and 32D cells were grown in RPMI containing 10% fetal calf serum (FCS) and 10% WEHI-conditioned media (WEHI-CM) as a source of IL-3. Stable mass populations of cells expressing *bcr-abl* transgenes were generated by retroviral infection as previously described (Cortez et al. 1995). BaF3 cell lines expressing v-Src and c-Raf-BXB (Canman et al. 1995) were grown in RPMI containing 10% FCS and 10% WEHI-CM. To induce the expression of v-Src and c-Raf-BXB, cells were treated with 75  $\mu$ M ZnCl<sub>2</sub> for 8 hr. To inhibit ubiquitin-dependent proteolysis, cells were treated with either 10  $\mu$ M lactacystin or 50  $\mu$ M LLnL for 8 hr. Cells were washed once with PBS and lysed directly in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol] for Western blot analysis.

### Anti-Abi antibodies

Rabbit polyclonal antibodies 5421 and 4575 anti-Abi were raised to a recombinant GST-Abi 2 $\Delta$ 1-100 fusion protein and a synthetic Abi 2 peptide (amino acids 318-329), respectively. Antibodies were affinity purified by standard techniques (Harlow and Lane 1988).

### RNA analysis

*abi 1* and *abi 2* mRNA levels were determined by RNase protection assays. Total RNA was isolated from BaF3 cells using TRIzol reagent (GIBCO BRL) or the RNeasy Mini Kit (Qiagen) as directed by each of the manufacturers. The protocols for these two methods were followed as directed. Antisense probes for *abi 1* and *abi 2* mRNA were generated by *in vitro* transcription with T3 RNA polymerase (Stratagene). *abi 1* probe was transcribed from a linearized template containing the T3 promoter

from the pPCR-Script Amp SK(+) plasmid (Stratagene) and a 244-nucleotide fragment of *abi 1* cDNA. *abi 2* probe was generated from linearized pBlueScript II SK +/- (Stratagene) plasmid containing a 435-bp fragment of *abi 2* mouse genomic DNA composed of a 48-bp intronic region and 397 nucleotides from a single exon. The probe for mouse GAPDH RNA was generated from the pTRI-GAPDH mouse linear fragment (Ambion) using T3 RNA polymerase. RNase protection experiments were carried out in accordance with instructions provided by the manufacturer (Ambion). Hybridization of probes to RNA protected the probes from digestion with RNase A plus T1 (Ambion). To detect *abi 2* by RNase protection, 30 µg of total RNA was used per lane. Twenty micrograms of RNA was used for *abi 1* and 10 µg of RNA was used for *GAPDH*. After digestion, protected probes for *abi 1*, *abi 2*, and *GAPDH* were analyzed in separate lanes of 6% acrylamide/urea gels. <sup>35</sup>S-labeled protected fragments were detected by autoradiography.

#### Analysis of *Abi* expression in bone marrow cells

P185-expressing primary mouse bone marrow cells were generated by retroviral infection as described (Cortez et al. 1995). Mouse bone marrow was obtained by flushing the femurs of 3- to 4-week-old male BALB/c mice (Charles River Labs) with IMDM containing 2% FCS and either was used for retroviral infection followed by G418 selection as described (McLaughlin et al. 1989), or processed through two rounds of ammonium chloride lysis, lysed in 2× SDS sample buffer and used directly for Western blot analysis using antibodies against the Abl kinase domain (8E9, Pharmingen) or Abi-2. Bone marrow samples from leukemia patients were obtained from the SWOG Human Tissue Bank (University of New Mexico) or the Duke Human Tissue Bank (Duke University Medical Center). Normal bone marrow was obtained after informed consent from patients undergoing autologous bone marrow transplantation for nonhematological malignancies. After Ficoll density gradient centrifugation (Sigma), light density human bone marrow cells were resuspended in PBS containing 0.01 mg/ml aprotinin (Boehringer Mannheim), 5 mM benzamide (Sigma), and 1 mg/ml AEBSF ("Pefabloc SC" Boehringer Mannheim), then lysed with 2× SDS sample buffer for Western blot analysis. Mouse myeloid and mouse and human bone marrow samples were normalized to give roughly equal levels of Abl immunoreactivity.

#### In vitro degradation assay

<sup>35</sup>S-labeled human Abi 2 was produced using RRL and plasmid pT7T3-Abi 2 with a coupled transcription-translation kit (Promega). The transcription-translation reaction was performed at 30°C for 120 min in the presence of [<sup>35</sup>S]methionine, as indicated by the manufacturer. To produce unlabeled p185Bcr-Abl, the transcription-translation reaction was performed using plasmid pGEMp185Bcr-Abl in the presence of a complete amino acid mix. Protein stability was analyzed by incubating 3 µl of <sup>35</sup>S-labeled Abi 2 in 50 µl of degradation mix [33% RRL, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1 mM ATP, and 2 mM methionine] at 37°C in the presence or absence of 3 µl of p185Bcr-Abl-containing lysate. Where indicated, 1 mM ATP was substituted with 2 mM ATPγS. The reaction was stopped by addition of an equal volume of 2× SDS sample buffer and analyzed by SDS-PAGE.

#### Affinity precipitation

Purification of His-tagged ubiquitin expressed transiently in Bosc 23 cells was performed following the method of Treier et

al. (1994). Briefly, 48 hr after transfection cells were lysed with 1 ml of GTN buffer per 60-mm dish [6 M guanidinium-HCl, 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM imidazole, 0.1% TX-100]. The lysate was sonicated with a microtipped sonifier at setting 4 for 20 sec to reduce viscosity. Fifty microliters of Ni<sup>2+</sup>-NTA-agarose beads (Qiagen) was added and mixed for 4 hr at room temperature. The beads were successively washed with the following solutions (pH 8.0): 1 ml of GTN; 1 ml of 8 M urea, 20 mM Tris-HCl, 200 mM NaCl, 0.1% TX-100; 1 ml of 8 M urea, 20 mM Tris-HCl, 1 M NaCl, 0.1% TX-100; 1 ml of 4 M urea, 20 mM Tris-HCl, 200 mM NaCl, 0.1% TX-100; 1 ml of 1 M urea, 20 mM Tris-HCl, 200 mM NaCl, 0.1% TX-100; and 1 ml of 20 mM Tris-HCl, 200 mM NaCl, 10 mM imidazole, 0.1% TX-100. The bound His<sub>6</sub>-tagged ubiquitin substrate complexes were analyzed by SDS-PAGE and Western blotting.

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